Frost tolerance of various *Pinus* species and hybrids

by

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature …Fanele Mabaso……..... Date: March 2017

ABSTRACT

Pinus species covers a large area in South African Forestry and are utilised by forestry companies for pulp, paper and sawlog products to achieve financial returns. Although *P. patula* is the most popular commercial *Pinus* species, studies have shown that field trials of *P. patula* are affected by frost after establishment, and the introduction of many pests and diseases, such as *Fusarium circinatum*. Other *Pinus* species such as *P. tecunumanii* LE and HE have been crossed with *P. patula* and used to replace plantings of *P. patula* since these species have increased tolerance to frost and *F. circinatum*.

The study objectives were to review and develop a reliable laboratory screening technique to assess frost tolerance of a range of *Pinus* pure species and hybrid families planted in South Africa. In-field climatic data was collected to construct a 24-hour circadian model, mimicking *in vivo* (day and night) temperature fluctuations to be simulated *in vitro* with electrolyte leakage and whole-tree freezing techniques. Rooted cuttings from a range of genotypes supplied by Sappi were tested *in vitro* at different target temperatures to determine their frost tolerance. These genotypes included pure species (*P. patula* seedlings and cuttings, *P. tecunumanii* LE, *P. tecunumanii* HE, *P. oocarpa*, *P. taeda*, *P. caribaea*, *P. elliottii*, *P. maximinoi* and *P. greggii*), three interspecific hybrids (*P. patula* x *P. tecunumanii* LE, *P. patula* x *P. tecunumanii* HE, and *P. elliottii* x *P. caribaea*) and a three-way cross (*P. patula* x (*P. patula* x *P. oocarpa*).

The results indicated that pure species *P. greggii*, *P. elliottii*, *P. patula* seedlings and cuttings, *P. tecunumanii* HE and *P. taeda* were more frost tolerant than other *Pinus* pure species employed in this study. In addition, the interspecific hybrids of *P. patula* x *P. tecunumanii* HE were more frost hardy than *P. patula* x *P. tecunumanii* LE.

There is variation in frost tolerance of PPTH families, therefore, a more comprehensive factorial mating design with more PTH families need to be screened in future studies. Also the number of replications need to be improved from six to 10 to limit experimental errors. *In vitro* screening for frost tolerance must be done before the establishment of field trials to determine the temperatures at which plants can survive and make informed decisions before planting.

OPSOMMING

Pinus spesies dek ‘n groot oppervlakte van Suid Afrikaanse Bosbou en word deur Bosbou maatskappye gebruik vir pulp, papier en saaghout om finansiële inkomstes te genereer. Alhoewel *P. patula* die mees aangeplante kommersiële *Pinus* spesies is, het vorige studies aangedui dit is vatbaar vir koue, asook peste en siektes soos *Fusarium circinatum,* na aanplantings. Ander *Pinus* spesies, bv. *P. tecunumanii* LE en HE is alreeds met *P. patula* gekruis en sal in die toekoms vir *P. patula* vervang aangesien dit beter koue en *F. circinatum* weerstand het.

Hierdie studie se doelwitte was om ‘n betroubare labaratorium tegniek te evalueer en te ontwikkel om die koue weerstand van *Pinus* spesies en hibried families, aangeplant in Suid Afrika, se koue weerstand te toets. Klimaatsdata is versamel om die veld toetstande te verteenwoordig en sodoende ‘n 24-uur circadian model op te stel. Hierdie model kan dag en nag *in vivo* temperature dus *in vitro,* met elektron lekkasie en heel-plant eksperimente, naboots. Bewortelde saailinge en steggies is vanaf Sappi verkry en *in vitro* getoets by verskillende teiken temperature om die koue weerstand daarvan te bepaal. Hierdie genotipies het ingesluit verskeie *Pinus* spesies (*P. patula* saailinge en steggies, *P. tecunumanii* LE, *P. tecunumanii* HE, *P. oocarpa*, *P. taeda*, *P. caribaea*, *P. elliottii*, *P. maximinoi* en *P. greggii*), drie interspesifieke hibriede (*P. patula* x *P. tecunumanii* LE, *P. patula* x *P. tecunumanii* HE, en *P. elliottii* x *P. caribaea*), en ‘n drie-ledige kruising (*P. patula* x (*P. patula* x *P. oocarpa*).

Resultate het aangedui dat die spesies *P. greggii*, *P. elliottii*, *P. patula* (saailinge en steggies), *P. tecunumanii* HE en *P. taeda* meer koue weerstandig as die ander spesies was. Verder was die interspesifieke hibried van *P. patula* x *P. tecunumanii* HE meer koue weerstandig as *P. patula* x *P. tecunumanii* LE.

Variasies in die koue weerstand van die PPTH families het aangedui dat ‘n meer volledige faktoriale teelontwerp wat meer PTH families insluit, in die toekoms ge-evalueer moet word. Die aantal herhalings moet ook van ses na 10 vermeerder word om verdere eksperimentele foute uit te skakel. *In vitro* skandeering van koue weerstand moet gedoen word voor aanplantings om sodoende die temperatuur waarby hierdie genotipes optimal sal funksioneer, te bepaal.

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LIST OF ABBREVIATIONS

EC	Electrolyte conductivity
EL	Electrolyte leakage
I_t	Injury index
RC	Relative conductivity
WPFT	Whole plant freeze test
List of species and hybrids	Table 3.1

CHAPTER ONE

INTRODUCTION

1.1. Background

The South African forestry plantation industry is distributed over a large land area from the Limpopo to the Western Cape Province (Smith *et al*., 2005). These plantation areas vary in soil type, while the climate ranges from cold dry conditions (Highveld) to warm sub-tropical conditions (Zululand), with summer and winter rainfall (DAFF, 2014). Many different *Pinus* species were evaluated over the last 100 years and suitable species were identified for the different site and climatic conditions. During the last 10 to 15 years, a number of pests and diseases have had a negative impact on the traditional commercial species and tree breeders have tested new species and hybrid combinations with increased disease tolerance. Many of these new species and hybrid combinations do not have the same level of frost tolerance compared to the original commercial species. Therefore, frost tolerance screening is critical to identify genotypes within these new species and hybrid combinations that will survive cold temperatures (Hodge & Dvorak, 2012).

The total forestry area in South Africa is about 1.3 million ha of which softwood species cover approximately 53% (DAFF, 2014). *Pinus patula* (337.467 ha) is the most widely planted softwood species, mainly due to its wide geographic adaptability, high volume growth and good wood quality suitable for both sawn timber and pulp (DAFF, 2014). Some of the other commercially important species are *P. elliotti*, *P. taeda*, *P. greggii*, *P. radiata* and *P. tecunumanii* (DAFF, 2014). These species are found in the winter (*P. radiata*, *P. taeda* and *P. elliottii*) and summer rainfall areas (*P. patula*, *P. elliottii* and *P. taeda*) (Dvorak, 1985).

Pinus patula has good frost tolerance but is highly susceptible to *Fusarium circinatum* (Mitchell *et al*., 2011). Some other *Pinus* species that have been evaluated by tree breeders, like *P. tecunumanii* and *P. oocarpa*, have been found to be tolerant to *F. circinatum*. When hybridising these species with *P. patula*, the hybrids have improved disease tolerance, but become less cold tolerant than *P. patula.* Hence, there is a need for frost tolerance testing of both pure species and hybrids. Therefore, a laboratory screening (*in vitro*) protocol to determine frost tolerance of *Pinus* pure species and hybrids used in breeding programmes need to be developed (Dvorak *et al*., 1996). Although various *in vivo* and *in vitro* cold tolerance testing techniques were evaluated during previous studies whether *in vitro* techniques

correlate with *in vivo* frost tolerance survival and rankings. Disadvantages of *in vivo* tests are that it is costly and results are obtained after a very long time compared to *in vitro* tests. Commercially deployed interspecific hybrids (*P. patula* x *tecunumanii* LE, *P. patula* x *tecunumanii* HE, *P. patula* x *oocarpa* and *P. elliottii* x *caribaea*) and pure species (*P. patula, P. greggii*, *P. oocarpa*, *P. maximinoi*, *P. taeda*, *P. caribaea* and *P. tecunumanii* LE and HE) were included in these screening experiments (Cerda, 2012).

1.2. Research objectives

The aim of this study is to develop a rapid, early nursery or *in vitro* technique to assess frost tolerance of *Pinus* species and interspecific hybrids. A range of *Pinus* species and interspecific hybrids important to the Southern African forestry industry were utilised to evaluate existing techniques and to determine a reliable technique, which correlates well with *in vivo* (field trials) results.

The following research questions were investigated:

- What is the frost tolerance of selected *Pinus* hybrid families and pure species?
- What *in vitro* techniques exist for screening frost tolerance?
    - Is the Electrolyte Leakage (EL) and Whole Plant Freeze Test (WPFT) techniques efficient to test frost tolerance *in vitro*?
    - Is there a correlation between EL and WPFT techniques?
    - Which of these two methods are best to rank *Pinus* species and interspecific hybrids for frost tolerance?
- Is there a difference in frost tolerance between pure *Pinus* species and among interspecific hybrid families?

1.3. Materials and methods

The EL and WPFT techniques were evaluated *in vitro* to screen 6-month old *Pinus* seedlings (pure species) and rooted cuttings (interspecific hybrids) for frost tolerance at four target temperatures (-3, -6, -9, and -12°C). The amount of tissue damage was assessed and the injury index (I_t) calculated for each selection as a measure of the plant's ability to tolerate frost at the different target temperatures.

1.4. Significance of the study

Early screening techniques of seedlings for frost tolerance can assist with the rapid, reliable low-cost screening of developed breeding material. This will shorten the breeding cycle and improve site species matching. It can also increase the return on research investment, as screening of large numbers of families under nursery or *in vitro* conditions is more cost effective than field trials. Furthermore, screening results can assist in selection of parental genotypes for hybrid mating designs with various genetic traits.

1.5. Thesis structure

This thesis consists of six chapters. Chapter one provides a general introduction, while Chapter two focuses on a literature study, including background information on techniques used to test frost tolerance. Systematic methodology is outlined in Chapter three. Chapter four contains the results obtained during this study while Chapter five is a discussion of the results. Chapter six gives an overview of the findings of the study as well as some recommendations.

CHAPTER TWO

LITERATURE REVIEW

2.1. Introduction

Temperature and frost susceptibility are two of the most important factors governing the distribution of trees worldwide (Sakai & Larcher, 1987). Previous studies indicated that trees differ in sensitivity to subfreezing temperatures (Hodge & Dvorak, 2012). For example, tropical and subtropical species may be damaged by frost (-3 to -14°C) while temperate and subtropical species (-7 to -28°C) are less affected by prolonged exposure to subfreezing temperatures (Hodge *et al*., 2012). This might be due to tree species that have evolved in frost prone areas, acclimatised and thus are adapted to or tolerate subfreezing temperature spells (Hodge & Dvorak, 2012).

The geographic distribution of species is strongly related to winter frost resistance (Sakai & Larcher, 1987, Larcher, 1995, Flint, 1972, George *et al*., 1974) as species have different frost resistance levels (Bannister, 1990). Climatic zones occupied by conifers are more restricted in the Southern Hemisphere (Sakai & Larcher, 1987). Therefore, species from cold areas are likely to have a higher frost tolerance, while species from warmer climates are more susceptible to frost (Sakai & Larcher, 1987). A classification of climate based on minimum temperatures has been used to correlate planting of species and frost tolerance (Sakai & Larcher, 1987). According to Cerda (2012) these climates are known as plant hardiness zones, which were first established in horticulture. Such zones are based on the lowest mean air temperatures of the coldest month given in degrees Celsius since their first usage in the USA.

Low temperature injury can occur in all plants, but the mechanisms and types of damage vary considerably (Levitt, 1980). Frost refers to a period of unusually cold weather as early spring or late winter temperature drops below the normal average minimum range (Colombo *et al*., 1984, Mitchell *et al*., 2011). Frost damage can have an effect on the entire plant or only a small part of the plant tissue, but both can affect product quality (Levitt, 1980). According to Levitt (1980), frost is independent of time, can occur for only short periods of time (2 to 24 hours), but depends on how fast the temperature drops and to what level it cools before freezing. Therefore, it can be divided into direct (ice crystals form inside the protoplasm of cells) or intracellular freezing and indirect (ice forms inside the plant but outside of the cells) or extracellular freezing (Levitt, 1980).

Cold hardiness on the other hand refers to the lowest temperature below the freezing point to which a seedling can be exposed without being damaged and is measured by the lowest temperature a plant can withstand (Glerum, 1976). This will vary greatly between different tree species. During a freeze, the level of hardiness at the time, the temperature, the rate of cooling and the duration of subfreezing temperatures can all affect the response of a plant (Aldrete *et al*., 2008). Therefore, this study will focus on frost tolerance and not cold hardiness.

2.2. Frost damage

There are two types of frost: spring frost that occurs when temperatures drop dramatically following warm temperatures experienced during summer; and winter frost occurs during winter and can affect trees of all ages resulting in dieback, growth deformities and cankers. Frost often affects trees with unhealed wounds, poorly established trees and juvenile trees planted close to winter (Sakai & Larcher, 1987). Tolerance to frost can vary with development stages of the tree, time of the year and ages of the tissue (Sakai & Larcher, 1987). Furthermore, degrees of frost tolerance at certain times of the year vary between the species, for example, Christersson *et al*. (1987) found that frost tolerance between *Picea abies* and *Pinus sylvestris* were significantly different.

Trees are very vulnerable to frost damage between bud break and shoot elongation (Levitt, 1980, Bolander, 1999). Frost damage can be in the form of needle tip scorching to whole plant scorching (Hodge *et al*., 2012) or foliage drop to recover in spring (Miller, 1993, Bolander, 1999). Within a day or two after a frost event, foliage and shoots can become limp, will start to fade from yellow to black, while leaves and shoots break off during the next few weeks (Miller, 1993, Bolander, 1999, Murray *et al*., 2012). Frost damage can also be observed on the cambium and roots (Murray *et al*., 2012). Damage caused is not just limited to growth reduction, loss of stem straightness, but can increase susceptibility of species to pest and diseases (Mitchell *et al*., 2011, Hodge *et al*., 2012). Different categories of damage can be identified. For example, Levitt (1980) developed four freeze-sensitivity categories, namely: tender (unable to withstand freezing temperature), slightly hardy, moderately hardy (slightly tolerant and susceptible) and very hardy (tolerant to frost).

Tree species have developed mechanisms in response to seasonal changes (Levitt, 1980, Sakai & Larcher, 1987), for example avoiding intracellular freezing and tolerate extracellular freezing (Repo *et al*., 2006). This can be maintained by depressing the freezing point with antifreeze proteins or by dehydration as less water can then freeze (Levitt, 1980, Sakai & Larcher, 1987). Frost tolerance,

therefore, is a complex trait which is influenced by several factors (Colombo, 1990), such as: bark thickness and wood hardiness, onset of dormancy, flower bud break, freezing tolerance of the buds, root hardiness, plant density and the effect of cultural practices (Levitt, 1980, Sakai & Larcher, 1987).

Genetic variation for frost tolerance and associated phenological traits do exist between different conifer species. These include phenology of bud break and growth cessation (Rehfeldt, 1984). In addition, it can also differ between populations, among and within families of a species (Colombo, 1990). For example, selection for growth rate can result in unfavourable correlated responses in bud phenology and frost hardiness (Colombo, 1990), or provenances with greater frost resistance have less growth potential (Rehfeldt, 1984). Therefore, the selection of appropriate species and provenances adapted to frost occurrence is a relevant factor to increase seedling survival and growth in reforestation programs (Rehfeldt, 1984).

In summary, Levitt (1980) developed two strategies to survive freezing temperatures:

- Freezing tolerance: plant tissue respond to low temperature stress by the loss of cellular water to extracellular ice. This results in the collapse of the cell, increase in the concentration of the cell sap and decline of the freezing point.
- Freezing avoidance: plant tissue avoids freezing stress by deep super cooling. This is a process where cellular water is separated from the dehydrated and nucleating effects of extracellular ice.

2.3. Frost-prone forestry areas in South Africa

The South African Forestry Industry stretches from Limpopo in the north to the Western Cape in the south (approximately 1.3 million ha). Variations in climate are evident from the cold and dry conditions of the Highveld, the warm sub-tropical Zululand to the winter rainfall in the Western Cape (DAFF, 2014). The frost prone areas include the Highveld region of Mpumalanga province and extends into the Midlands region of KwaZulu-Natal, where a high number of frost days occur annually (Figure 2.1). Therefore, a range of *Pinus* species (Table 2.1) have been planted historically to match these climatic conditions (Hodge & Dvorak, 2012).

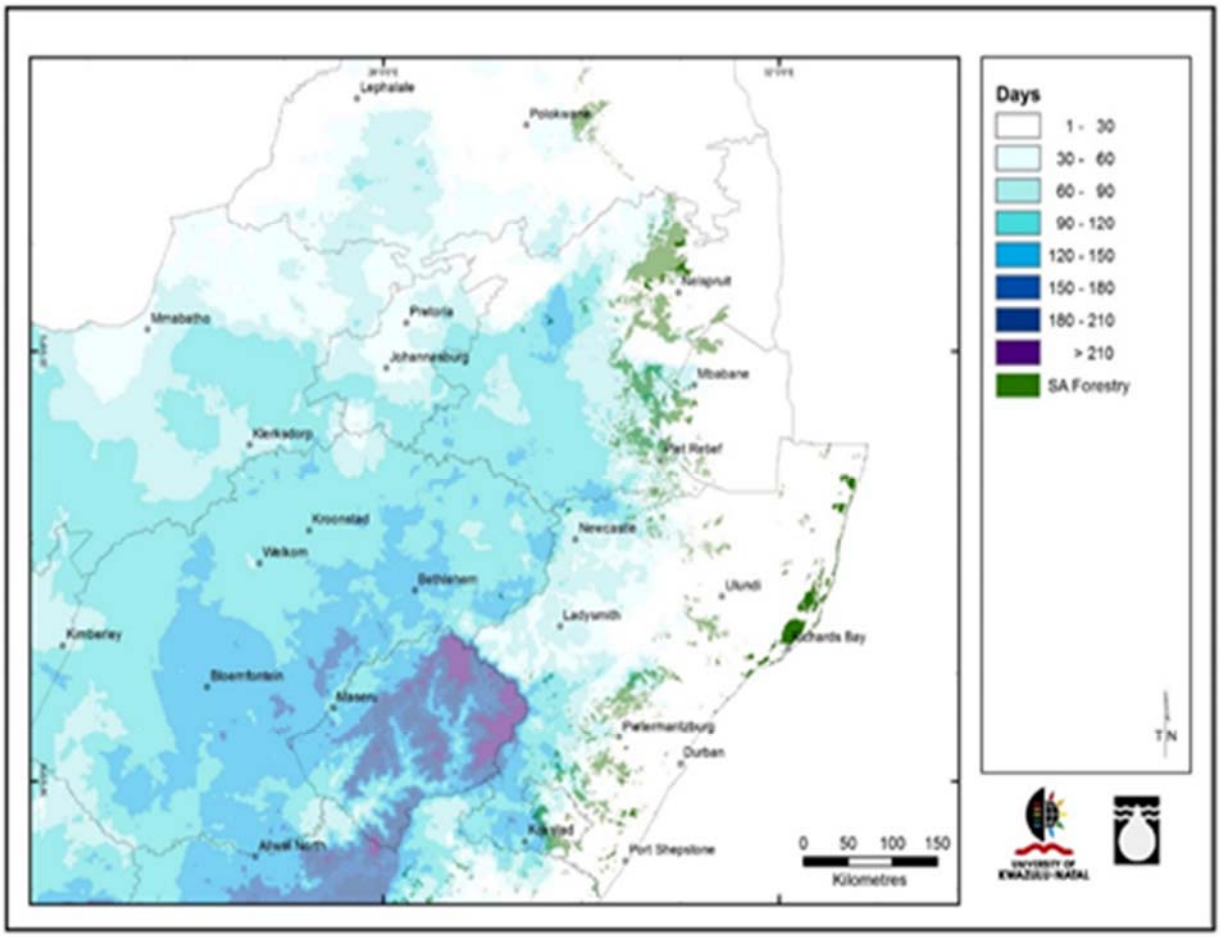

Figure 2.1: Frost-prone areas in South Africa (Schulze & Maharaj, 2007)

Currently, more effort and money is invested in developing new interspecific pine hybrids with superior growth, good wood quality, disease and frost tolerance (DAFF, 2014). Tropical (*P. caribaea*, *P. taeda*, *P. patula*, and *P. oocarpa*) and sub-tropical (*P. tecunumanii* and *P. maximinoi*) *Pinus* species planted in South Africa can experience occasional sub-freezing temperatures (-3 to -10°C) during the winter months (May to July) at higher altitudes (Mpumalanga and KwaZulu-Natal midlands) (DAFF, 2014). (Table 2.1). Interspecific hybrids with these frost prone species will further increase frost susceptibility. (Kanzler, 2007). Field trials in the Lowveld area (Spitskop and Wilgeboom) indicated that *P. tecunumanii* has good growth potential when planted in exotic plantations (Mitchell *et al*., 2012), this species has been used in South Africa as a interspecific hybrid partner with *P. patula* (Kanzler, 2007, Kanzler *et al*., 2014).

Table 2.1: Optimum growth site conditions for *Pinus* species planted in South Africa (Giutierrez & Donahue, 1987, Osorio, 2000, Dvorak 1985, Dvorak *et al*., 2009, Dvorak *et al*., 2000a, Dvorak, 1985, Gymnosperm database, 2016, Richard *et al*., 2016)

Latin name	**Common name**	**Native to**	**Altitude (m.a.s.l) and latitude**	**Climate**	**Advantages**	**Disadvantages**
P. patula	Mexican yellow pine	Mexico, northestern Oaxaca, Siera Madre	1490-3100 16-24°N	MAP: 1100-2500 MAT: less than 18°C (optimal between 12 to 17°C Cold hardiness: -10°C	Cold hardy, good wood quality.	Susceptible to *F. circinatum*.
P. greggii	Gregg's pine	Eastern Mexico	1100-2500 24-25°N	MAP: 600-1850 MAT: 13 to 15°C Cold hardiness: -18°C	Drought and cold tolerant, will hybridise with other pine species.	Performs poor on wet sites.
P. elliottii	Slash pine	George Town, Central Florida. North central Georgia and Alabama	800-1500 8-10°N	MAP: 700-900 MAT: less than 14°C (optimal from 17 to 22°C) Cold hardiness: unkown	Fire tolerant from a young age.	Lack of drought tolerance.
P. tecunumanii HE	Schwerdtfeger's pine	Guatemala, Chiapas, Mexico	1170-2900 14-17°N	MAP: 1150-2590 MAT: 15 to 18°C Cold hardiness: -3°C	Tolerant to frost.	Susceptible to *F. circinatum*.

Latin name	Common name	Native to	Altitude (m.a.s.l) and latitude	Climate	Advantages	Disadvantages
P. tecunumanii LE	Schwerdtfeger's pine	Belize (northern Guatemala), Honduras, Nicaragua	400-1650 12-17°N	MAP: 900-1600 MAT: 15 to 18°C Cold hardiness: 0°C	Tolerant to *F. circinatum.*	Susceptible to frost.
P. maximinoi	Thin-leaf pine	Mexico, Guatemala, northern Nicaragua.	600- 2400 20-24°N	MAP: 900-2400 MAT: 14 to 20°C Cold hardiness: -2 and -3°C	*F. circinatum* tolerance suitable for pulp and paper.	Susceptible to frost.
P. taeda	Loblolly pine	Southern United States (Georgia and Northern Florida)	0-400 17-38°N	MAP: 625-1250 MAT: less than 13°C (optimal from 15 to 24°C) Cold hardiness: -18 and -22°C	Fast growth rate, good wood properties.	No drought tolerance, lack of adequate growing season.
P. oocarpa	Mexican yellow pine	Mexico, Southern Sonora & Northern Nicaragua	200-2500 13-28°N	MAP: 800-2300 MAT: 16 to 26°C Cold hardiness: 0°C	Tolerant to *F. circinatum*.	Susceptible to frost.
P. caribaea	Caribbean pine	Central America & Mexico (Honduras, Belize, Nicaragua)	5-1000 12-28°N	MAP: 660-4200 MAT: 22 to 27°C Cold hardiness: 0°C	Good wood properties, easy to propagate as cuttings.	Susceptible to frost, pest and diseases.

The commercial planting of *Pinus* species is limited by their sensitivity to cold (lower) temperatures, rainfall and growth characteristics like stem straightness (Wormald, 1975). Currently, *P. patula* is the most widely planted *Pinus* species in South Africa due to the geographic distribution and pulp properties (DAFF, 2014). *Pinus patula* grows on approximately 340 000 ha of land which corresponds to slightly more than 50% of the total historical softwood plantation area in South Africa (Dvorak *et al*., 2000a). This species is mainly planted in the northern and southern regions of Mpumalanga, Eastern Cape and KwaZulu-Natal (Mitchell *et al*., 2012). There are two varieties, namely *P. patula* var. *patula* and *P. patula* var. *longipedunculata* (Figure 2.3). *Pinus patula* var. *patula* and some genotypes of *P. patula* var. *longipedunculata* from northern Oaxaca (Mexico) are cold tolerant and can withstand extremely low temperatures of -12 to -18°C (Wormald, 1975). Genotypes of *P. patula* var. *longipedunculata* from southern and western Oaxaca (Mexico) are, however, more susceptible to cold weather and suffers frost damage when planted in South Africa (Duncan *et al*., 1996). This thesis will focus on *P. patula* var. *patula* as it is planted commercially in South Africa.

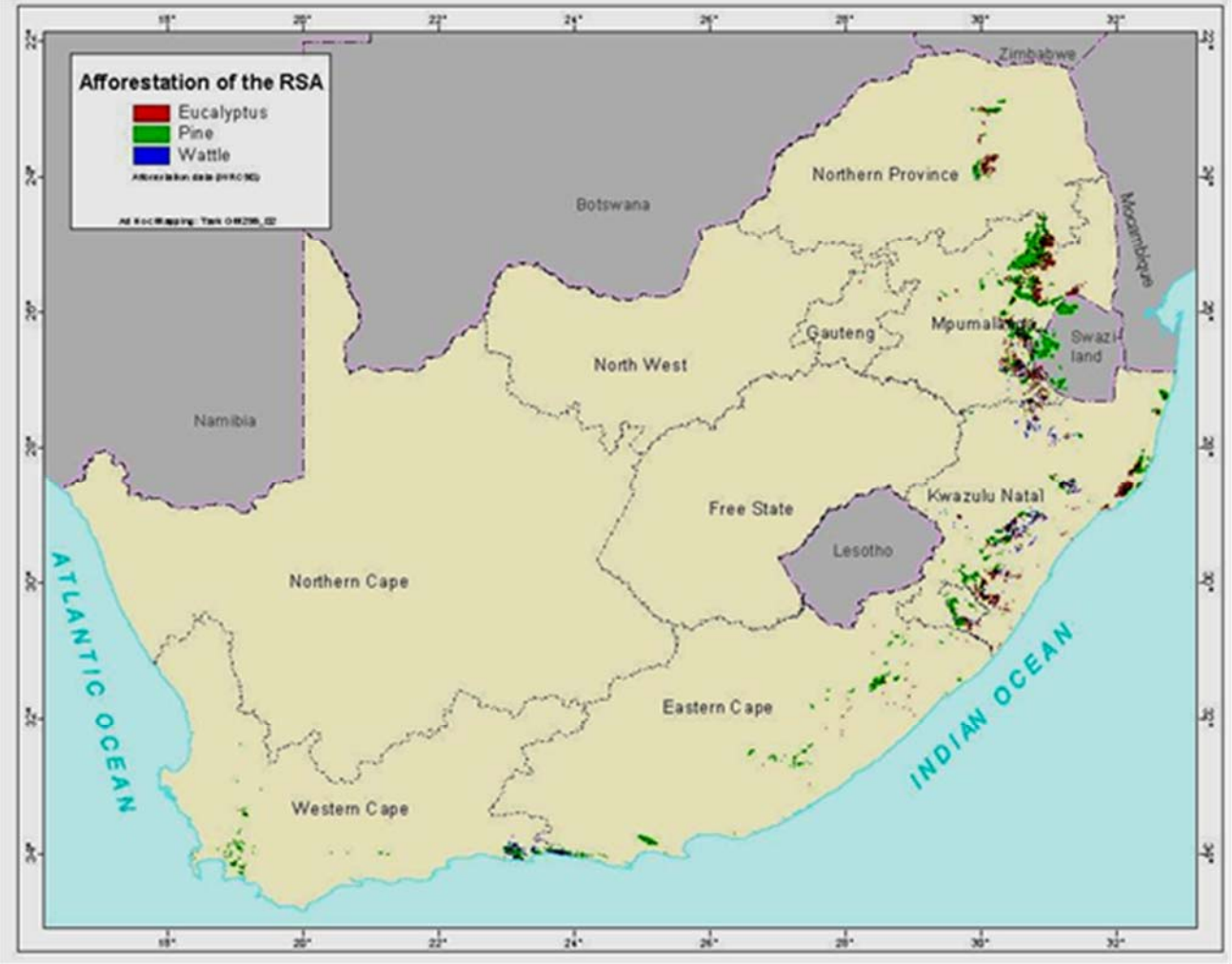

Figure 2.2: Areas planted with *Pinus* species in South Africa (Dvorak *et al*., 2000a).

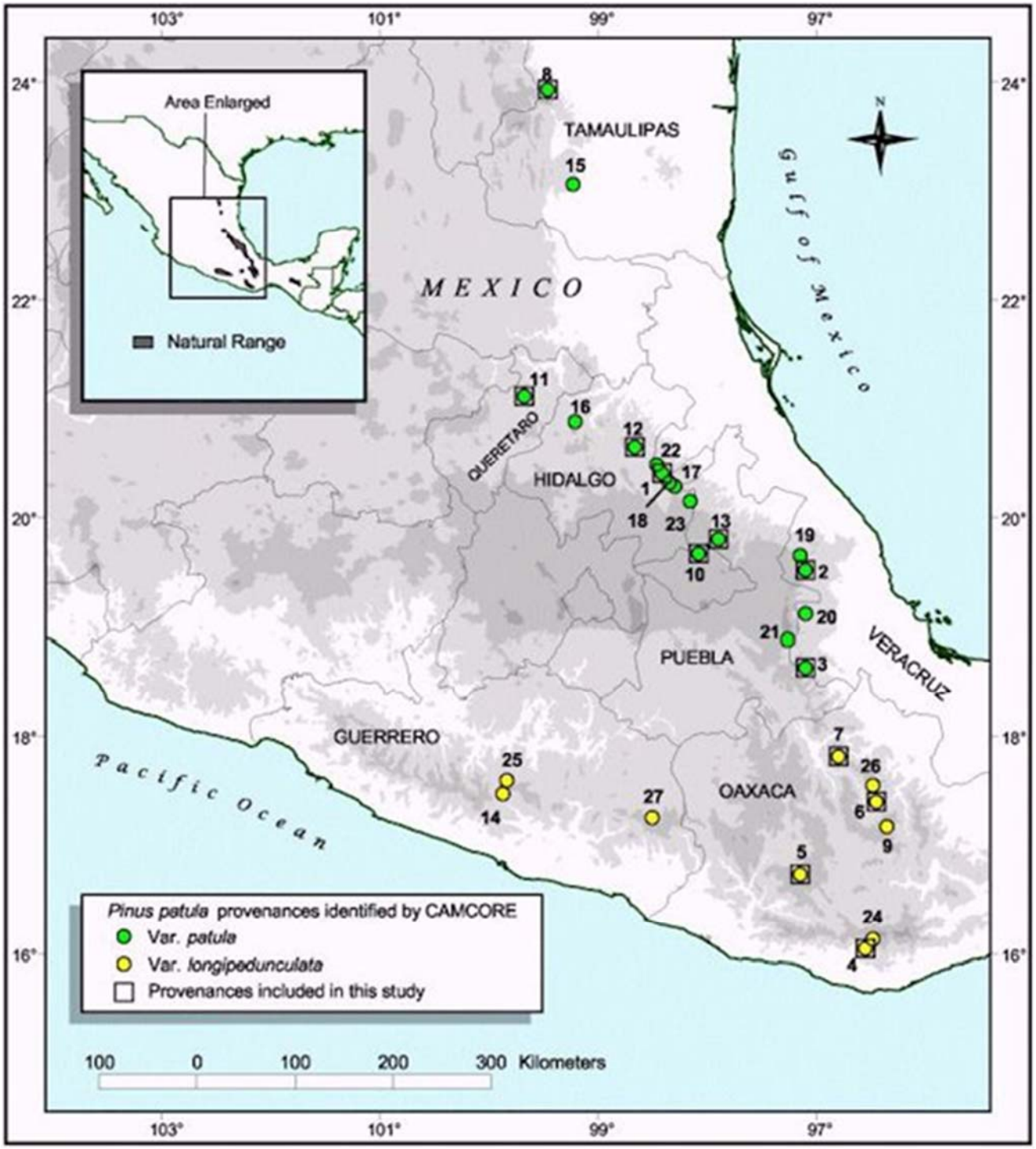

Figure 2.3: Map of the natural occurrence of *P. patula* in Mexico (Dvorak *et al*., 2000a).

2.3.1. *Pinus patula*

Pinus patula is a fast growing species (18 m^3 ha^{-1} yr^{-1}) and prefers higher altitude sites (1 490 to 3 100m.a.s.l.) where severe frosts and snow can occur (Mitchell *et al*., 2011). Previous studies indicated that this species is tolerant to frost but is highly susceptible to *F. circinatum* (Mitchell *et al*., 2011). Therefore, *P. patula* needs to be hybridised with *F. circinatum* tolerant *Pinus* species for improved tolerance to *F. circinatum*, extending the planting area and decreasing the economic losses due to poor site species matching (Tibbis *et al*., 1991, DAFF, 2014). Other potential species that can be planted in high frost prone areas in South Africa is *P. greggii var. greggii*, (Volker *et al*., 1994), but it is also susceptible to *F. circinatum*.

Interspecific *Pinus* hybrids between *P. patula* and *P. tecunumanii* (both high and low elevation) appear to be a suitable replacement for sub-temperate and temperate sites (Kietzka, 1988, Gapare *et al*., 2001). Furthermore, *P. tecunumanii* hybridises easily with *P. patula* and might improve the growth rate, ease of vegetative propagation, wood properties, frost and *F. circinatum* tolerance (Mitchell *et al*., 2011). *Pinus patula* has also been successfully hybridised with *P. elliottii*, *P. greggii*, *P. taeda*, *P. maximinoi*, *P. caribaea* and *P. oocarpa* (Hodge & Dvorak, 2012). Some of these hybrids combinations could offer acceptable frost and *F. circinatum* tolerance, while other hybrid combinations will be more susceptible. Therefore, significant effort is invested in the development of pine hybrids that have superior growth and wood properties, improved disease tolerance and acceptable frost tolerance.

2.4. Methods for measuring frost tolerance in conifers

Frost tolerance can be measured by exposing plant tissue to controlled freezing temperatures, and quantifying tissue damage by one or more methods (Burr *et al*., 1990). It is important to adhere to well defined, standardised testing protocols and evaluation methods in order to accurately estimate frost tolerance and compare data from different testing methods (Tinus *et al*., 1985). Differences between tests include the type of information provided, the precision and accuracy of the information, speed with which results were available and the plant material required to perform the test (Tinus *et al*., 1985).

The most common methods employed for testing frost tolerance in conifers are:

- Shoots (cut to a certain length) are pre-treated at low temperatures to ensure maximum hardening before being exposed to a series of successively lower temperatures (-7, -14 and -21°C) for periods of 4 to 16 hours (Sakai & Larcher, 1987). Climent *et al*. (2009) have found

that primary needles were significantly more sensitive to freezing than secondary needles in some *Pinus* species.

- Visual assessment of frost damage to plant tissues of shoots and intact plants (Stanley & Warrington, 1988, Timmis, 1976).
- Electrolyte leakage (EL) method is based on *in vitro* stress of leaf tissues and is a subsequent measurement of EL into an aqueous medium (Sakai & Larcher, 1987). This technique has also been applied to quantify damage to cell membranes in various abiotic stress conditions such as low and high temperatures (-3 to -7°C) (Garty *et al*., 2000).
- A range of chemical tests like neutral red or 2, 3 triphenyl tetrazolium chloride (TTC) investigating water potential gradient across tissues can also be used. The colour reactions caused by neutral red and TTC can distinguish dead from live cells (Garty *et al*., 2000).
- Measurements of electrical impedance on plant stems before and after freezing can help to quantify tissue damage (Blazich *et al*., 1974). This method involves the taking of an electrical impedance measurement with a 1 kHz impedance bridge before exposing seedlings or seedling parts to freezing temperatures followed by another measurement after the freezer treatment has been completed (Glerum, 1995). Although this method is rapid and non-destructive, many factors can complicate the interpretation of impedance measurements (Repo *et al*., 2000).

These methods have been developed to understand the many thermodynamic, physiological, anatomical and biochemical features of plants involved in acclimation and de-acclimation to freezing temperatures (Burr *et al*., 1990). In addition, these methods evolved from rapid monitoring of frost tolerance to successful production of conifer nursery stock for reforestation (Burr *et al*., 1990).

2.4.1. EL method

Early assessment of frost tolerance relies only on field data or freezing chamber experiments (Tibbits *et al*., 1991). An *in vitro* method (Injury Index) can now be used to measure frost tolerance under controlled conditions, enabling more reliable and repeatable results. Injury Index (I_t) measures the needle or shoot damage in terms of electrolyte conductivity (EC) of pine needles exposed to below zero temperatures (Anisko & Lindstrom, 1995, Hodge *et al*., 2012). EC is a measure of plant material's ability to conduct electrical current (Krzyzanowski & Vieira, 1999).

Recording the amount of EL after the stress treatment provides an estimate of the tissue injury (Hodge *et al*., 2012) and are expressed as a percentage of total EL from a heated or frozen (killed) sample (Flint *et al*., 1967). However, unfrozen samples need to be included as a control (Flint *et al*., 1967). Therefore,

EL values are indices of injury (Flint *et al*., 1967) and range between 0 to 100% (Aldrete *et al*., 2008). However, poor leaching of electrolytes may cause problems in interpretation of leakage data from well-acclimated woody plants (Anisko & Lindstrom, 1995). Hodge *et al*. (2012) considered values more than 60% as dead (susceptible to frost).

The method is fast and reliable and can determine frost damage within a few days by providing objective, precise, reputable and quantitative data. Small amounts of plant tissue can be screened, for example needles. The equipment needed for the screening is inexpensive and the method is non-destructive as only a small portion of needles, are harvested (Hodge *et al*., 2012).

However, the method can cause problems with interpreting the temperature curves as it does not distinguish the points at which the plants tissue is damaged (Burr *et al*., 1990). During sampling, the errors made can decrease precision and reputability of results (Aldrete *et al*., 2008). In addition, fertilisation can increase ion concentration as genetic differences in nutrient uptake and ion diffusion rate can be affected by cuticle properties (Osmocote, 2016). Lastly, membrane properties may be affected by previous stress (Hodge *et al*., 2012).

2.4.2. Whole Plant freeze Test

Whole plant freeze testing (WPFT) is the standard method used to assess frost tolerance of seedlings. This involves freezing of the entire seedling (including root section) in a controlled temperature chamber (Colombo *et al*., 1984, Burr *et al*., 1990). The seedlings are then maintained under optimum growing conditions until visible signs of injuries are evident. This test simulates testing under *in vivo* conditions and is called the browning test (Glerum, 1995).

As the whole intact plant is exposed to the test temperature, it allows for the interaction among tissues and organs within the plant as recovery and injury progressed to determine the biological and operational viability (Timmis, 1976). It is also considered the most accurate test to simulate estimation of *in vivo* frost tolerance (Burr *et al*., 1990). Due to the long duration time of the test, the results are only evident in 7 to 10 days; and this could lead to delayed seedling growth in the forestry nursery. In addition, destructive sampling and poor precision with small sample sizes are possible disadvantages that can occur (Lopez-Upton & Donahue, 1995).

2.4.3. Alternative methods

Differential Thermal Analysis (DTA) technique can also be used to measure frost tolerance of some tree species and is related to the capacity of super-cooling (Burke *et al*., 1976). Super-cooling refers to the cooling of a solution below the freezing point prior to ice formation. This method has been used in *Abies*, *Acer*, *Carya*, *Fraxinus*, *Gleditsia*, *Juniperus*, *Larix*, *Picea*, *Pseudotsuga*, *Quercus*, *Turga* and *Ulmus* species (Tinus *et al*., 1985). In non-super cooling genera such as *Pinus*, however, DTA does not indicate frost tolerance (Burke *et al*., 1976).

General disadvantages to frost tolerance screening:

In vitro screening of frost tolerance can have several shortcomings and might be inaccurate predictors of *in vivo* frost survival (Burr *et al*., 1990). This might be due to age of plant material used in experiments as frost tolerance might differ between juvenile and mature plant tissues (Sakai & Larcher, 1987). As small sections of a juvenile seedling are used, this can result in unreliable indicators of *in vivo* behaviour due to ice nucleation temperatures of excised plant parts generally decrease because of super-cooling (Ashworth & Ristic, 1993). It is also important to correlate artificial screening results with survival assessments carried out in field trials where the whole tree is exposed to cold temperatures. However, field trials have a limitation in that extreme weather conditions occur randomly (Sakai & Larcher, 1987). Therefore, some trials might escape exposure to critical frost conditions and might lead to a misinterpretation of the suitability of the genotypes in certain field trials.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Introduction

This study consisted of three separate experiments (pilot, EL and WPFT) with five steps each (Figure 3.1). The steps included growing the genetic material (seedlings and rooted cuttings), needle harvest (nursery), the freeze test (four different target and control temperatures) for both EL and WPFT, and data analysis.

Seedlings from various economically important *Pinus* species and hybrids were selected for frost tolerance screening. Acronyms used for the pure species and hybrids screened during this study are summarised in Table 3.1, with different families indicated by numbers. Pure species screened included *P. patula* (seedlings and cuttings), *P. oocarpa*, *P. tecunumanii* high (HE) and low elevation (LE), *P. elliottii*, *P. caribaea*, *P. greggii*, *P. maximinoi* and *P. taeda*. There are two varieties of *P. greggii*, the southern variety *P. greggii* var. *australis* and the northern *P greggii* var. *greggii*. In this study only *P. greggii* var. *greggii,* which is the most frost tolerant of the two varieties, and the most tolerant pine species available in South Africa, was used. Interspecific hybrids were developed according to a factorial mating design (Table 3.2) between *P. patula*, *P. tecunumanii* LE, *P. tecunumanii* HE and *P. patula* x *P. oocarpa* and were propagated as rooted cuttings. A total of 10 pure *Pinus* species and 26 hybrid families were screened. This included 20 *P. patula* x *P. tecunumanii* LE, three *P. patula* x *P. tecunumanii* HE, two *P. patula* x (*P. patula* x *P. oocarpa)* three-way crosses, and one *P. elliottii* x *P. caribaea* hybrids.

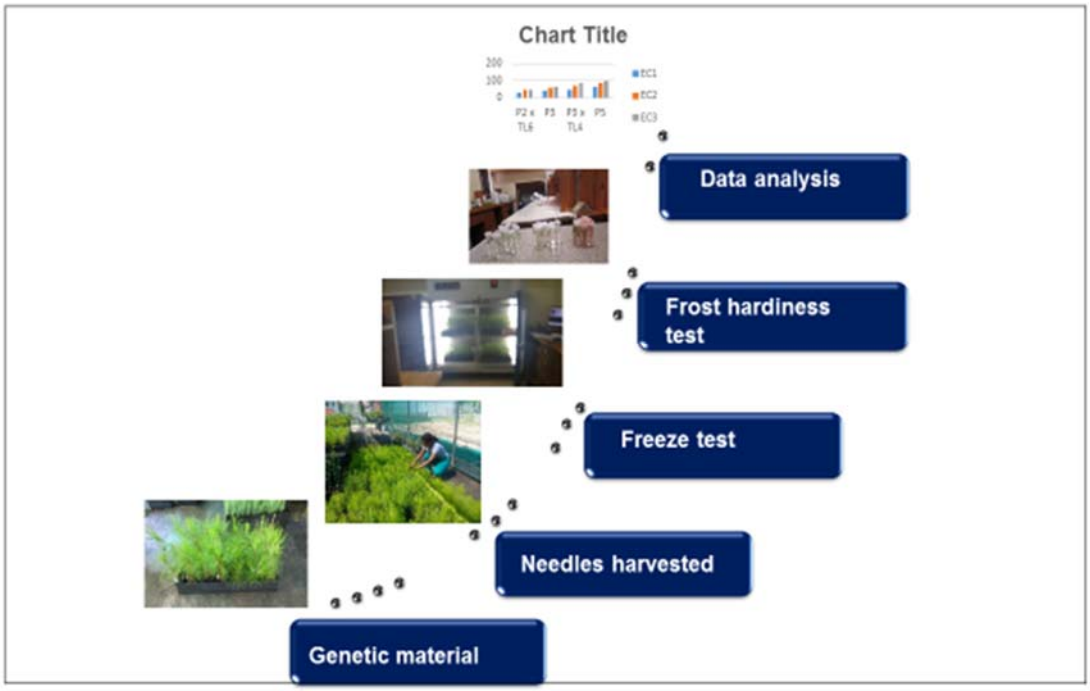

Figure 3.1: Outline of work plan employed in this study

Table 3.1: Abbreviated coded of pure *Pinus* species and interspecific hybrids screened in this study

Pure species/hybrid	**Abbreviation**
P. patula	patula seed
P. patula families	P_1 to P_7
P. patula cuttings	patula cuttings
P. oocarpa	oocarpa
P. greggii	greggii
P. maximinoi	maximinoi
P. elliottii	elliottii
P. taeda	taeda
P. caribaea	caribaea
P. tecunumanii HE	PTH
P. tecunumanii HE families	PTH_1 and PTH_3
P. patula x *tecunumanii HE*	$PPTH_1$ and $PPTH_2$
P. tecunumanii LE	PTL
P. tecunumanii LE families	PTL_1 to PTL_7
P. patula x *tecunumanii* LE	$PPTL_1$ to $PPTL_7$
P. elliottii x *caribaea*	PECH
P. patula x *oocarpa*	PPOH
P. patula var. *patula* x *oocarpa*	$PPPOH_1$ and $PPPOH_2$

Table 3.2: Factorial mating design for interspecific hybrids between *P. patula*, *P. oocarpa*, *P. tecunumanii* low elevation (PTL) and *P. tecunumanii* high elevation (PTH) families screened during this study.

Patula	**PTL**						**PTH**		**PPOH**	
	PTL_1	**PTL_2**	**PTL_3**	**PTL_4**	**PTL_5**	**PTL_6**	**PTH_1**	**PTH_3**	**$PPOH_1$**	**$PPOH_2$**
P_1						X	X	X	X	X
P_2	X	X				X				
P_3			X	X	X	X				
P_4										
P_5		X	X	X	X		X			
P_6	X		X	X		X				
P_7	X	X	X			X				

3.2. Seedling growth conditions

Pinus seeds were sown and cuttings produced in a commercial forest nursery by Sappi Research. Unigro 98 black plastic trays with a capacity of 98 seedlings and inserts (7 x 14mm) were used for both seedlings and cuttings. Trays were filled with a commercial nursery growth medium (mixture of coya and perlite). Before the 90: 10 (perile: coya) growth medium was prepared, 250 granules of Osmocote per gram was added in order to reduce risks of pest and diseases. Osmocote is a coated NPK fertilizer that releases nitrogen, phosphate and potassium and trace elements over a pre-chosen period of time (Osmocote, 2016). For optimum germination, Unigro trays were placed in a growth tunnel at 30ºC for 24 hours and fertilised three times a week with nursery blue mixture or Osmocote irrigation water.

The seed sown were from both controlled crosses and open pollination collections from *Pinus* species and interspecific hybrids (Table 3.1). Seedlings (obtained from control crosses) were first established in hedges to produce shoot cuttings. Seed were stored in a fridge between 1 and 2 years before sowing to limit mixing of seed between selections and to increase germination percentage as opposed to seed stored at room temperature (Colombo *et al*., 1995). The seedlings were watered twice a day in summer and once per day during winter. The goal was to raise seedlings that were approximately 25cm in height at the time of needle harvest (Hodge & Dvorak, 2012).

Seed from the open and cross pollination *Pinus* species and interspecific hybrid collections (Table 3.1) were sown directly. The seed were sown one month after the cuttings were set to account for expected differences in growth rate. Six months after planting, the cuttings and seedlings were transported to Stellenbosch, Western Cape (cool temperate zone), for the commencement of experiments. In Stellenbosch the plants were kept in the nursery for one month to acclimatise where they received irrigation by sprinklers twice a day.

3.3. Climatic data

Three months climatic data (June to August 2015) from a frost prone area, Pinewoods plantation, close to Howick in KwaZulu-Natal (30°2230556"S, -29°4822222"E) at 1340m.a.s.l. was used to develop a 24-hour circadian model to represent *in vivo* conditions. Comparisons were done by plotting daily and hourly averages (Theron, 2000, Nel, 2002).

3.4. Screening experiments

3.4.1. Pilot experiment:

The aim was to find the most optimal target temperatures at which needles and the whole plants will be screened. Data loggers (EL-USB-2) were calibrated and used to monitor temperatures during all of the experiments. Fresh primary healthy needles (approximately 8 or 9) of three selections (greggii, PTL, and PPPOH) were collected from the nursery and placed in labelled paper bags. Needles were cut into 3cm units with sterilised laboratory blades. The needles were then put into glass test tubes and weighed with an electronic scale (Ohaus SPJ601 Prorable Scale**),** ensuring needles had the same length and weight. Samples from each of the three selections were placed in glass tubes as a control (4ºC) and target temperatures of -5, -10 and -15ºC respectively (Hodge & Dvorak, 2012).

Three samples from each of the three *Pinus* families were placed in a freezer at target temperatures (-5 for 3 hours, -10 for 6 hours and -15ºC for 3 hours) to expose the plant tissues to low temperatures (Hodge & Dvorak, 2012). The samples were then moved from the freezers and ionized distilled water (9ml) was added into each glass tube before samples were placed in a shaker (100rpm) for 16 hours. Samples were removed from the shaker and EC_1 was measured (Hanna DiST® EC Tester, HI98304) to determine the EL for the frozen treatment. Glass tubes with samples were placed in the oven at 85ºC for two hours to completely kill the plant tissue, and EC_2 was measured (Hodge & Dvorak, 2012).

3.4.2. EL experiments:

From the pilot experiment, results indicated that the target temperatures of -5, -10 and -15ºC were too extreme as the survival rate was low. Furthermore, the time (in hours) interval differed between the target temperatures and can create unnecessary error in the data. Therefore, the target temperatures were narrowed down to -3, -6, -9 and -12ºC, complementing results from section 3.3. Also, the time (hours) at each temperature interval were kept constant (Figure 3.2). A total of 36 samples (10 pure *Pinus* species and 26 interspecific hybrids) were screened. Young healthy needles were collected, cut into 3cm units, weighted and put into glass test tubes (Figure 3.3). Control samples (unfrozen) were put in the fridge at 4ºC for 24 hours; however, frozen samples were also placed at 4ºC for 3 hours (Figure 3.2). Frozen samples were then placed in the growth chamber (Scientific Manufactures series 1400 LTIS) at 0ºC for 1 hour, followed by 6 hours at the selected target temperatures (-3, -6, -9, -12ºC). Afterwards samples were placed again at 0ºC for an hour followed by 4ºC for 3 hours.

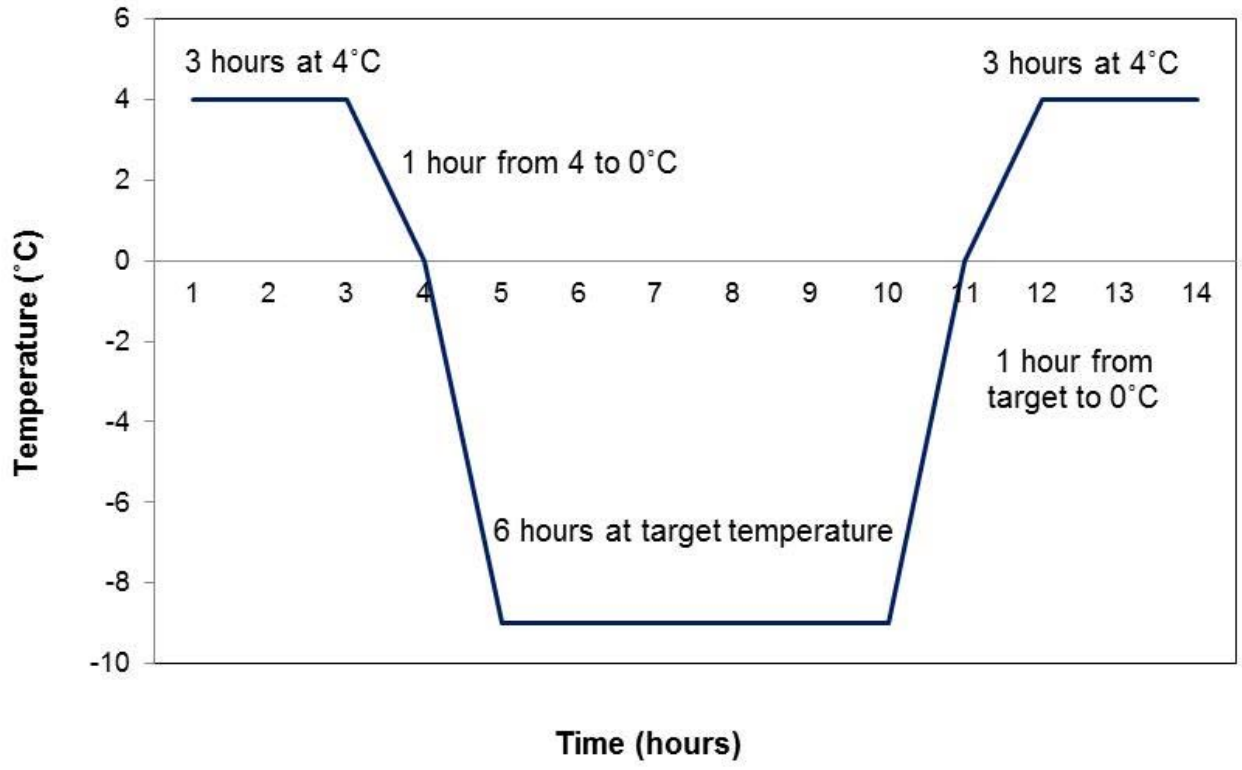

Figure 3.2: The diagram illustrating the target temperature protocol used to determine for both EL and WPFT experiments

Twenty-five ml ionized distilled water was added to the glass tubes and the samples were placed in a shaker for 16 hours (100rpm) before measuring EC_1 (HANNA EC/TDS HI 991300). Afterwards, samples were placed in the oven at 85ºC for 2 hours and EC_2 was measured (Figure 3.3). Samples were placed back into the shaker for another 16 hours at 100rpm and EC_3 was measured. The EC was

measured three times to ensure that the plant tissue was completely mixed with the distilled water after the heat treatment (Hodge & Dvorak, 2012). This experiment was repeated twice (total of six replications per sample) to verify results.

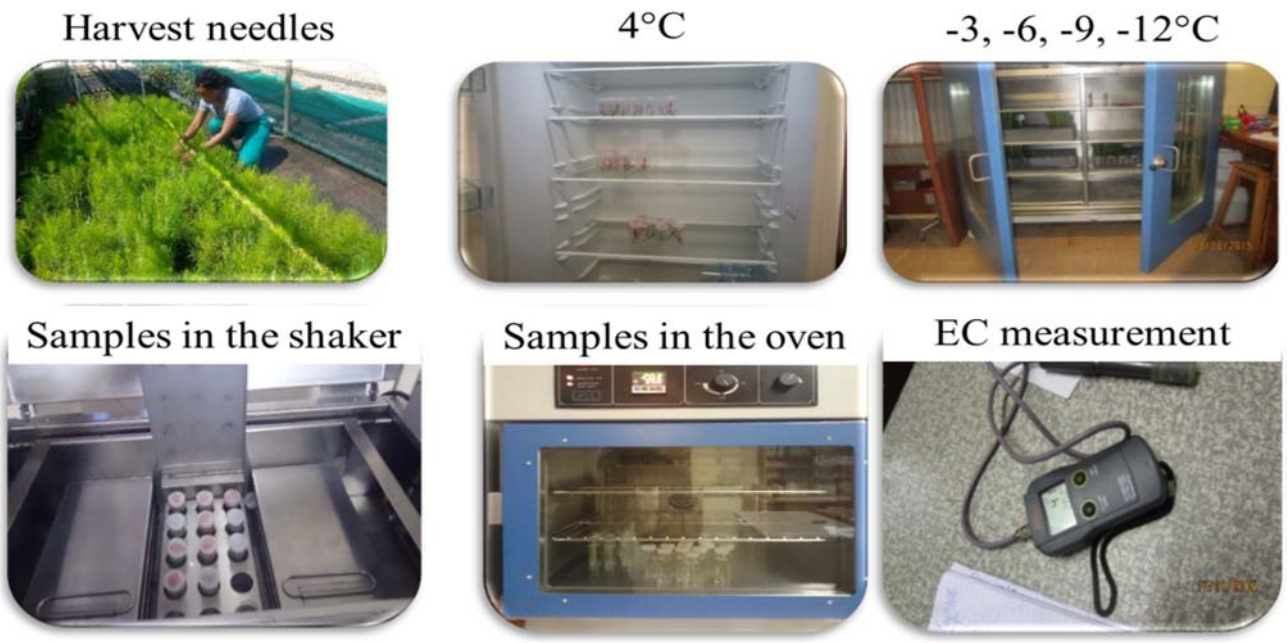

Figure 3.3: Process (clockwise) of determine EC and frost tolerance for both EL and WPFT experiments

3.4.3. WPFT experiments:

Three replications (seedlings) from each of the 36 *Pinus* families were selected in the nursery and used for the *in vitro* WPFT experiment. The freezing protocol for the EL method was employed for the WPFT experiment (Figures 3.2 and 3.3) in order to compare results between the two experiments (Burr *et al*., 1990). Seedlings were moved back to the nursery for scoring (Bannister & Lee, 1989) after the freezing protocol. The seedlings were observed for survival up to 7 days after exposure to the target temperatures (South *et al*., 1993). Scoring was done by evaluating the extent of seedling injury and colour of the seedling tissue (Figure 3.4). For scoring of plant survival, the scores of 1 to 3 were used. Green indicated no damage (score of 1), yellow was intermediate (score of 2) and brown indicated severe damage (score of 3) (Bannister, 1990).

Figure 3.4: Examples of WPFT screening indicating dead (A), intermediate (B) and healthy seedlings (C) in the nursery

3.5. Statistical analysis

The experiment employed a completely randomised block design with a factorial treatment structure: 10 pure species and 26 hybrids with 6 replications each. An experimental unit (selection x temperature x replication) consisted of 216 treatments per target temperature (-3, -6, -9 and -12˚C). For each family replicate and temperature run, the I_t was calculated as reported by Flint *et al*. (1967). The I_t is calculated to correct inherent differences among species or replications as the amount of EL that takes place in the control (unfrozen) and frozen samples (Flint *et al*., 1967). Therefore, average relative conductivity (RC) and I_t values were calculated for each species across replicates and target temperatures (Hodge & Dvorak, 2012).

The RC and I_t of the frozen and control treatments were calculated as follows (Flint *et al*., 1967, Verwijst & von Fircks, 1994):

$$RC = \left(EC_1 / EC_2\right) \times 100$$

Where:

EC_1= is the EC of the sample before heat treatment

EC_2= is the EC of the sample after the heat treatment to completely kill the tissue.

$$I_t = \frac{100\left(RC_{frozen} - RC_{control}\right)}{1 - RC_{control}}$$

Where I_t is the injury index resulting from exposure to temperature (t).

An analysis of variance (ANOVA) for each temperature unit and selection were conducted by PROC GLM with SAS EG software, system for windows 10). A Shapiro Wilk test for normality was conducted before the results could be assumed reliable. A Fischer's Least Significant Difference test (LSD) with p = 0.05 (5%) was used to compare treatment means (Shapiro Wilk, 1965, Ott & Longnecker, 2001,

SAS, 2016). The sources of variation were partitioned into selections, replications within temperatures, species and temperatures, as well as the interactions of temperatures and species.

$$I_t = \mu + Y_i + L_j + YL_{ij} + \varepsilon_{ij}$$

I_t= injury index, general mean (μ), effect of temperature (Y_i), effect of selections (L_j), interaction of temperature and selections (YL_{ij}) and error ($\mathcal{E}_{ij}$).

Correlations between the EL and WPFT experiments were calculated using the Pearson correlation coefficient. Raw data for all experiments (EL and WPFT) were used to test whether there is a correlation between the EL and WPFT techniques, pure species and hybrids, as well as between pure species and hybrid family across the target temperatures (-3, -6, -9 and -12°C).

CHAPTER FOUR

RESULTS

4.1. Climatic data:

In vivo maximum temperature was logged between 14:00 and 15:00 (21°C), while minimum was reported between 07:00 and 08:00 (-3°C), resulting in an approximate 25°C temperature range (Figure 4.1). Temperature was below zero for approximately 8 hours between 00:00 and 08:30. In the raw data, temperature dropped once to -13°C (three hours), which can cause more damage than the minimum temperature of -3°C.

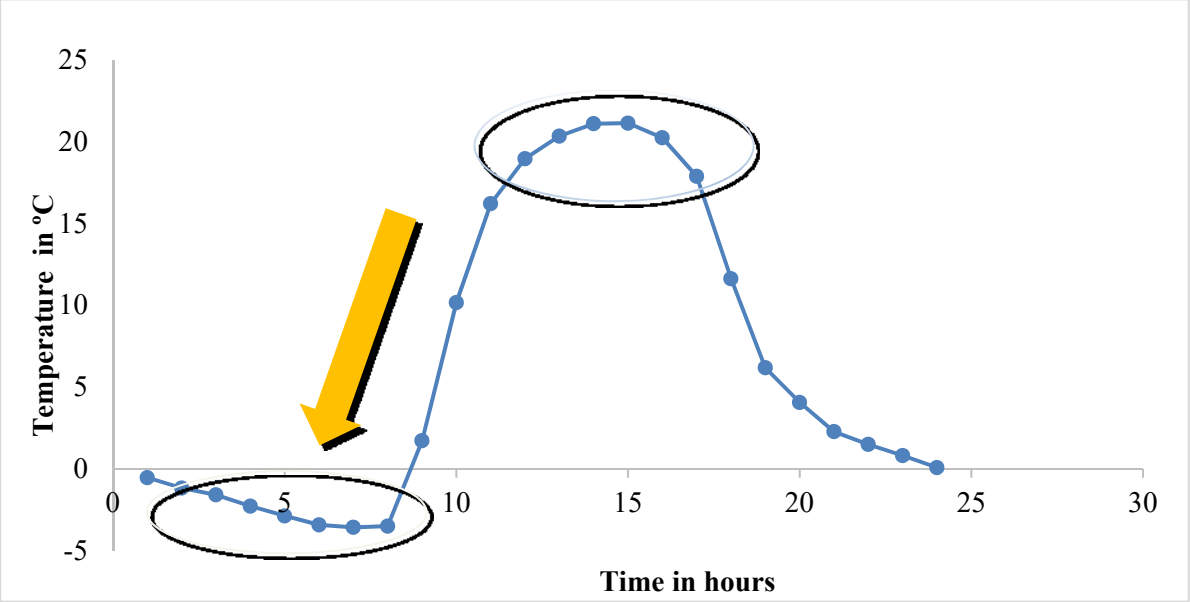

Figure 4.1: The 24-hour circadian model representing *in vivo* temperatures as measured at Pinewoods (KwaZulu-Natal)

4.1.1. Frost tolerance screening

Results of the experiments are discussed as follows:

- Pilot experiment: evaluating the target temperatures by screening three selections (PPPOH, greggii and PPTL) at -5, -10 and -15°C.
- EL: determine the I_t of 26 selections (interspecific hybrids and pure species) at target temperatures (-3, -6, -9 and -12°C).
- WPFT: determine the I_t of 36 selections (interspecific hybrids and pure species) at target temperatures (-3, -6, -9 and -12°C).

- Although the significance level of 0.05 was used, the *p* and r -values are indicated in brackets where significant differences apply.
- For each experiment, three statistical analysis were done to compare (a) constant temperatures, (b) fluctuations in temperature and (c) species versus fluctuations in temperatures.

For consistency between the results of the experiments, I_t is expressed as a percentage according to three main categories. An I_t of 0 to 40% or 1 represents frost tolerance; 40 to 60% or 2 represents moderate tolerance to frost; and 60 to 100% or 3 is considered susceptible to frost.

4.2. Pilot experiment:

4.2.1. EL test with needle material

4.2.1.1 Constant and fluctuations in temperature

Comparison of species per target temperature simulating constant temperatures indicated that all the species were susceptible to frost at -15°C (Figure 4.2). Greggii, PPTL and PPPOH did not differ significantly at all three target temperatures. When comparing species across target temperatures simulating fluctuations in temperatures, greggii had a low I_t value at -5°C indicating tolerance to frost (Table 4.1). However, at -10°C it was moderately tolerant to frost and susceptible to frost at -15°C. PPPOH was susceptible to frost at all target temperatures, while PPTL was moderately tolerant to frost at -5°C and susceptible at -10 and -15°C.

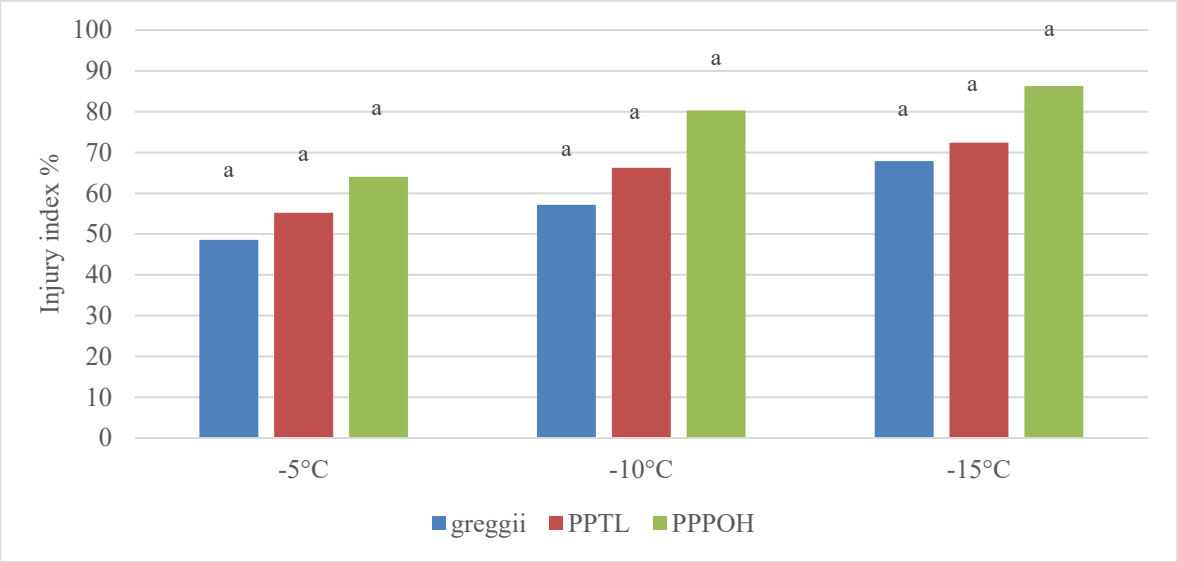

Figure 4.2: Comparison of the mean I_t for all the selections screened during the pilot experiment (EL) per target temperature (standard deviation bars with the same letters does not differ significantly, $p = 0.14$, $r^2 = 0.48$, $n = 3$)

Table 4.1: Comparison of the mean I_t of the selections (from left to right n rows) across target temperatures screened during the pilot experiment (EL) at target temperatures of -5, -10 and -15°C (n = 3)

Selection	**Target temperature (ºC)**		
	-5 (57.4 ± 6.7 [a])	**-10** (70.0 ± 10.1 [b])	**-15** (77.0 ± 8.9 [b])
greggii	[2] 48.6 ± 0.1 [a]	[2] 57.1 ± 0.2 [a]	[3] 67.9 ± 2.3 [a]
PPTL	[2] 55.2 ± 0.3 [a]	[3] 66.2 ± 0.6 [a]	[3] 72.4 ± 2.8 [a]
PPPOH	[3] 64.0 ± 1.2 [a]	[3] 80.3 ± 1.9 [a]	[3] 86.3 ± 2.9 [a]

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

Means in rows with the same letter does not differ significantly

4.2.1.2. Species versus fluctuations in temperatures

Comparison of the I_t of selections screened during the EL experiment across the three target temperatures (-5, -10 and -15°C) indicated no significant differences (p = 0.14, r^2 = 0.48, n = 3) between the three selections (Figure 4.3). Frost tolerance, ranked from high to low, was greggii, PPTL and PPPOH.

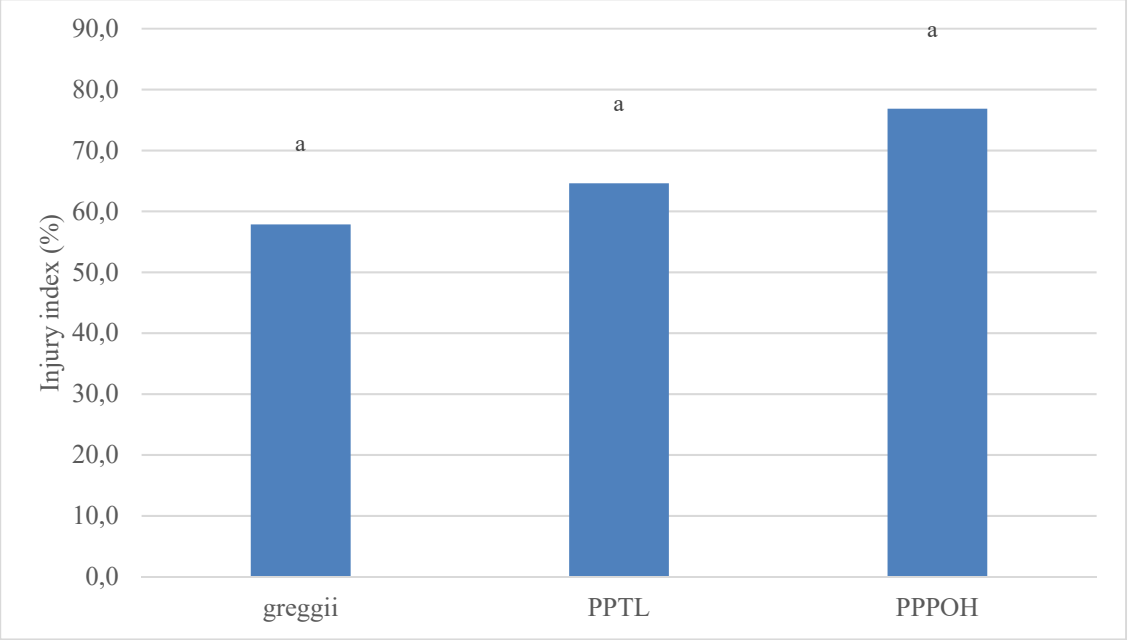

Figure 4.3: Comparison of the mean I_t of the selections screened during the pilot experiment (EL) across all three target temperatures -5, -10 and -15°C (standard deviation bars with the same letters does not differ significantly, p = 0.14, r^2 = 0.48, n = 3)

Grouping of species for the WPFT was the same as for EL (Table 4.2) with greggii being the most tolerant. PPPOH was susceptible to frost at all three target temperatures, while PPTL was susceptible at -10 and -15°C. Furthermore, frost damage to seedlings was more evident at -10 and -15°C, while -5°C showed differences between selections. Results from both the EL and WPFT indicated that the target temperatures of -5, -10 and -15°C were too severe. Therefore, the target temperatures were adjusted to -3, -6, -9 and -12°C to complement data obtained from *in vivo* data loggers.

Table 4.2: Comparison of the mean I_t for selections screened (left to right in rows) during the pilot experiment (WPFT) at target temperatures of -5, -10 and -15°C (n = 3)

Selections	**Target temperature (ºC)**			**Average**
	-5	**-10**	**-15**	
greggii	2	2	3	2
PPTL	2	3	3	2.7
PPPOH	3	3	3	3
Average	3.5	2.7	3	

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

4.3. The EL experiment with needles:

4.3.1. Pure species

4.3.1.1. Constant temperatures

As the lowest recorded *in vivo* temperature was -3°C (Figure 4.1) and the I_t obtained at -9 and -12°C were in general more than 50%, only significant differences between species at -3 and -6°C will be reported on except where mentioned otherwise (Figure 4.4). There were significant differences (p = 0.01 and r^2 = 0.31) between pure species at -3 and -6°C. Greggii differed significantly (p = 0.13, r^2 = 0.65) from the other species. Elliotti, patula (seed and cuttings), teda and PTH did not differ significantly from each other but differed from the other species. Maximinoi, PTL, oocrpa and caribaea did not differ significantly from each other but differed from the other species at -3°C. At -6°C greggii, elliottii, patula (seed and cuttings), taeda, PTH and maximinoi did not differ significantly from each other but differed from the other species. PTL, oocarpa and caribaea did not differ significantly from each other but differed from other species.

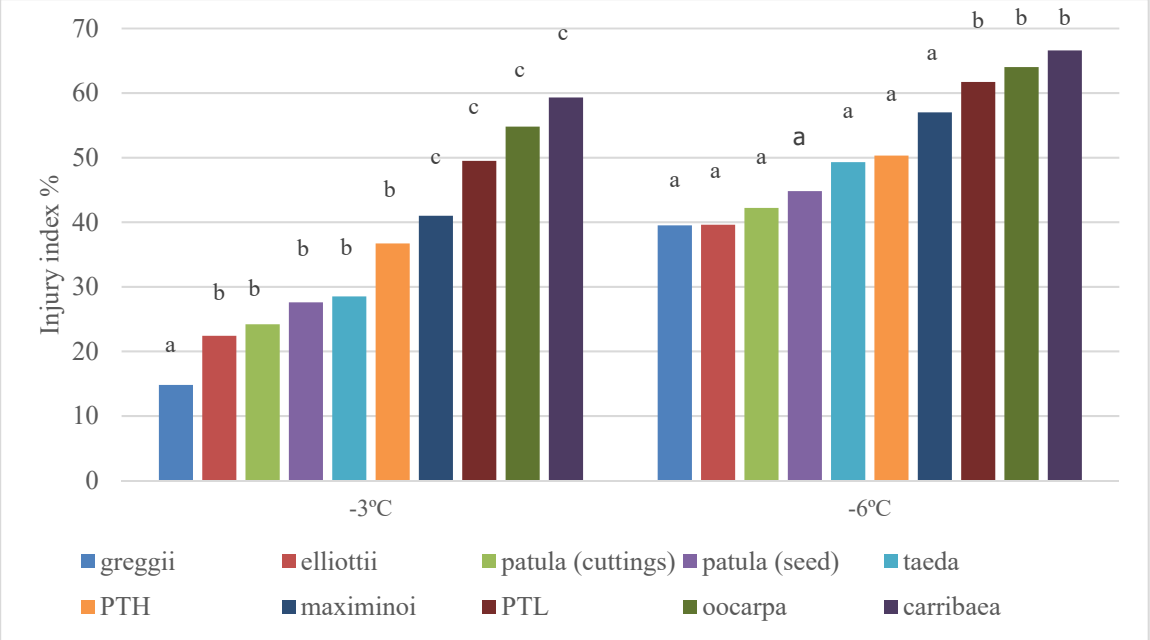

Figure 4.4: Mean I_t for all the pure species screened during the EL experiment per target temperatures (standard deviation bars with the same letters does not differ significantly, $p = 0.13$, $r^2 = 0.65$, $n = 6$)

Table 4.3: Mean I_t for all the pure species screened during the EL experiment at -3 and -9°C (standard deviation bars with the same letters does not differ significantly, $p = 0.13$, $r^2 = 0.65$, $n = 6$)

Species name	Target temperature (ºC)	
	-3 (35.7± 14.9) [a]	**-6** (51.8 ± 9.9) [b]
greggii	[1] 14.8 ± 2.9 [a]	[1] 39.5 ± 1.2 [a]
elliottii	[1] 22.4 ± 0.9 [b]	39.6 ± 21.2 [a]
patula (cuttings)	[1] 24.2± 6.0 [b]	[2] 42.2 ± 16.7 [a]
patula (seed)	[1] 27.6± 3.1 [b]	[2] 42.3 ± 7.9 [a]
taeda	[1] 28.5± 2.2 [b]	[2] 44.8 ± 1.9 [a]
PPTH	[1] 36.7± 4.3 [b]	[2] 49.8 ± 1.3 [a]
maximinoi	[2] 41.0 ± 4.1 [c]	[2] 55.5 ± 14.9 [a]
PPTL	[2] 49.5 ± 3.3 [c]	[3] 62.0± 1.5 [b]
oocarpa	[2] 54.8 ± 1.8 [c]	[3] 64.0 ± 8.3 [b]
caribaea	[2] 59.3 ± 1.4 [c]	[3] 66.6 ± 0.8 [b]

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

Means in rows with the same letter does not differ significantly

4.3.1.2. Fluctuations in temperatures

Comparing species across target temperatures, caribaea had the highest I_t value at all four target temperatures; therefore, it was the most susceptible to frost (Table 4.3, 4.4). Patula cuttings had a slightly better tolerance than patula seed at -3 and -6°C, most likely due to the fact that cuttings are more woody plants than seedlings, while greggii and elliottii were frost tolerant at -3 and -6°C. Maximinoi and oocarpa performed the same at -3 and -6°C (moderate tolerance), while taeda was tolerant to frost at -3°C and moderately tolerant at -6°C. PTH had a better survival rate than PTL at all four target temperatures.

Table 4.4: Comparison of the mean I_t for each selection of the pure species (left to right in rows) across target temperatures screened during the EL experiment at target temperatures of -3, -6, -9 and -12°C (n = 6)

Species name	**Target temperature (°C)**			
	-3 (35.7± 14.9 [a])	**-6** (51.6 ± 9.9 [b])	**-9** (64.7 ± 8.4 [c])	**-12** (69.1± 8.0 [c])
greggii	[1] 14.8 ± 2.9	[1] 39.5 ± 1.2	[2] 52.2 ± 2.0	[2] 56.5 ± 11.3
elliottii	[1] 22.4 ± 0.9	[2] 39.6 ± 21.2	[3] 63.5 ± 7.9	[3] 65.4 ± 14.1
patula cuttings	[1] 24.2± 6.0	[2] 42.2 ± 16.7	[2] 57.2 ± 4.6	[3] 64.6 ± 26.0
patula seed	[1] 27.6± 3.1	[2] 42.3 ± 7.9	[2] 59.3 ± 11.4	[3] 65.7 ± 28.1
taeda	[1] 28.5± 2.2	[2] 44.8 ± 1.9	[3] 70.2 ± 5.9	[3] 75.2 ± 13.9
PPTH	[1] 36.7± 4.3	[2] 49.8 ± 1.3	[2] 59.2 ± 7.4	[3] 61.2 ± 0.9
maximinoi	[2] 41.0 ± 4.1	[2] 55.5 ± 14.9	[3] 63.6 ± 2.3	[3] 67.6 ± 7.1
PPTL	[2] 49.5 ± 3.3	[3] 62.0 ± 1.5	[3] 69.8 ± 1.9	[3] 73.6 ± 4.1
oocarpa	[2] 54.8 ± 1.8	[3] 64.0 ± 8.3	[3] 71.8 ± 0.7	[3] 76.4 ± 0.6
caribaea	[2] 59.3 ± 1.4	[3] 66.6 ± 0.8	[3] 79.3 ± 3.9	[3] 83.6 ± 3.3

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

Means in the rows with the same letter does not differ significantly

4.3.1.3. Species vs fluctuations in temperatures

Comparison of the I_t of selections screened during the EL experiment across -3 and -6°C indicated significant differences (p = 0.18, r^2 = 0.32) between pure species (Figure 4.5). Frost tolerance was ranked from high (greggii, patula (cuttings and seed), elliotii and taeda) moderate (PTH, maximinoi, PTL and oocarpa) to low (caribaea).

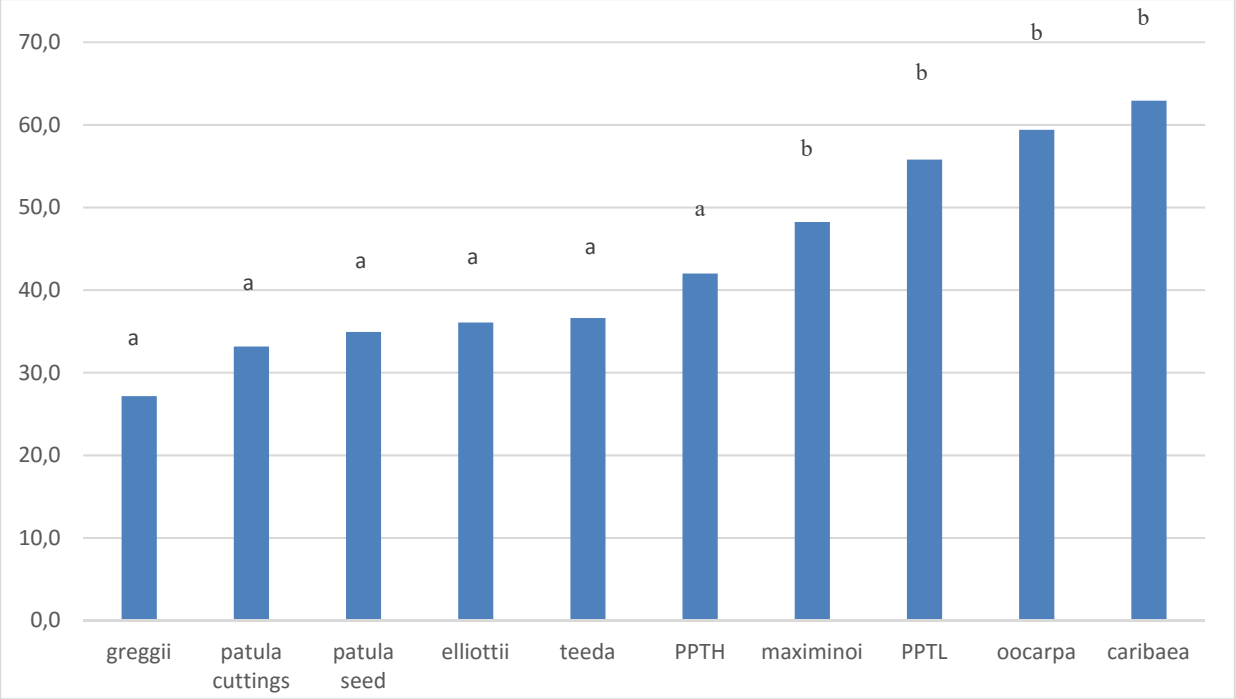

Figure 4.5: Comparison of the mean I_t for all the selections of pure species screened during the EL experiment across the target temperatures of -3 and -6°C (standard deviation bars with the same letter does not differ significantly, $p = 0.13$, $r^2 = 0.65$, $n = 6$)

4.3.2. Patula seed versus patula cuttings

4.3.2.1. Constant temperatures

The I_t at -9 and -12°C were also in general more than 50%, therefore, only significant differences of -3 and -6°C were be reported on except where mentioned otherwise (Figure 4.6). Patula cuttings were compared to patula seed to determine whether the I_t will differ at -3 and -6°C. Patula families P_1 to P_7 were also cuttings. There were no significant differences in frost tolerance between patula families at both -3 and -6°C. at -3°C all patula famiels were tolerant to frost ($I_t < 40\%$) and at -6°C all patula parents had a moderate tolerance to frost ($I_t < 40\%$).

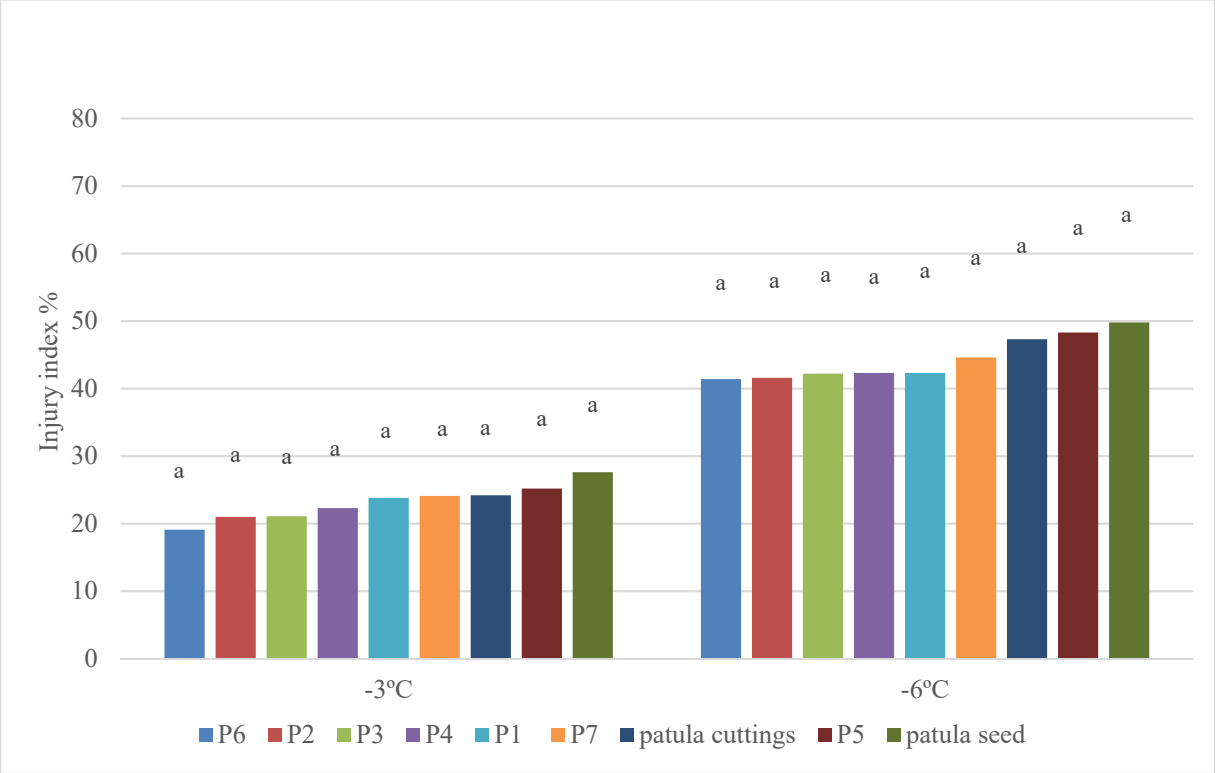

Figure 4.6: Mean I_t for all the pure species screened during the EL experiment per target temperatures of -3 and -6°C (standard deviation bars with the same letters does not differ significantly, p= 0.18, r^2= 0.059, n = 6)

Table 4.5: Mean I_t for all the patula families screened during the EL experiment per target temperatures of -3 and -6°C (standard deviation bars with the same letters does not differ significantly, p= 0.18, r^2= 0.059, n = 6)

	Target temperature (ºC)	
Seed or cuttings	**-3 (23.3 ± 2.8) [a]**	**-6 (44.7 ± 3.1) [B]**
P6	[1] 19.1 ± 5.1 [a]	[2] 41.4 ± 7.9 [a]
P2	[1] 21.0 ± 4.7 [a]	[2] 41.6 ± 14.2 [a]
P3	[1] 21.1 ± 3.6 [a]	[2] 42.2± 5.6 [a]
P4	[1] 22.3± 4.9 [a]	[2] 42.3 ± 12.4 [a]
P1	[1] 23.8 ± 4.9 [a]	[2] 42.3 ± 16.7 [a]
P7	[1] 22.4 ± 6.0 [a]	[2] 44.6 ± 9.2 [a]
patula cuttings	[1] 24.2 ± 6.0 [a]	[2] 47.3 ± 16.7 [a]
P5	[1] 25.2 ± 6.5 [a]	[2] 48.3 ± 13.3 [a]
patula seed	[1] 27.6 ± 3.1 [a]	[2] 49.8 ± 7.9 [a]

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

Means in rows with the same letter does not differ significantly

4.3.2.2. Fluctuations in temperatures

Comparison of patula families across target temperatures indicated that all patula families were tolerant to frost at -3 and -6°C. At -9 and -12°C (Table 4.5, 4.6), patula familes ranged from moderate tolerance to susceptible to frost. Patula parents (P1 to P7) were tolerant to frost at -3°C and had a moderate tolerance at -9°C. Patula seed and cuttings were tolerant to frost at -3°C and moderate tolerant at -6°C.

Table 4.6: Comparison of the mean I_t of the patula seed versus cuttings (left to right in rows) across target temperatures screened during the EL experiment at target temperatures of -3, -6, -9 and -12°C (n = 6)

	Target temperature (ºC)			
Seed or cuttings	**-3 (23.3 ± 2.8) [A]**	**-6 (44.7 ± 3.1) [B]**	**-9 (61.0 ± 4.3 [c])**	**-12 (61.2 ± 7.5 [c])**
P1	[1] 23.8 ± 4.9	[2] 42.3 ± 16.7	[2] 55.7 ± 13.4	[2] 59.3 ± 37.2
P2	[1] 21.0 ± 4.7	[2] 41.6 ± 14.2	[2] 58.4 ± 24.9	[3] 61.1 ± 16.9
P3	[1] 21.1 ± 3.6	[2] 42.2 ± 5.6	[3] 63.5 ± 7.9	[3] 65.4 ± 14.1
P4	[1] 22.3± 4.9	[2] 42.3 ± 12.4	[3] 61.8 ± 10.6	[3] 65.4 ± 30.2
P5	[1] 25.2 ± 6.5	[2] 48.3 ± 13.3	[2] 59.8 ± 15.1	[3] 66.7 ± 22.1
P6	[1] 19.1.5± 5.1	[2] 41.4 ± 7.9	[3] 63.0± 10.8	[3] 67.1 ± 8.4
P7	[1] 24.1 ± 7.1	[2] 44.6 ± 9.2	[3] 57.2 ± 4.6	[3] 64.6 ± 26.0
patula cuttings	[1] 24.2 ± 6.0	[2] 47.3 ± 16.7	[3] 60.9 ± 8.3	[3] 63.6 ± 17.4
patula seed	[1] 27.6 ± 3.1	[2] 49.8 ± 7.9	[2] 59.8 ± 11.4	[3] 65.7 ± 28.1

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

Means in rows with the same letter does not differ significantly

4.3.3.3. Species versus fluctuations in temperatures

Significant differences were evident at different target temperatures (Table 4.4 and Figure 4.6) for the different patula families. However, when comparing the patula families, seedlings and cuttings across -3 there were no significantly differences between patula families (Figure 4.7). Patula familes ranged from tolerant (P6, P2, P3, P4, P1 and P7) to moderate tolerance (patula cuttings, P5 and patula seed).

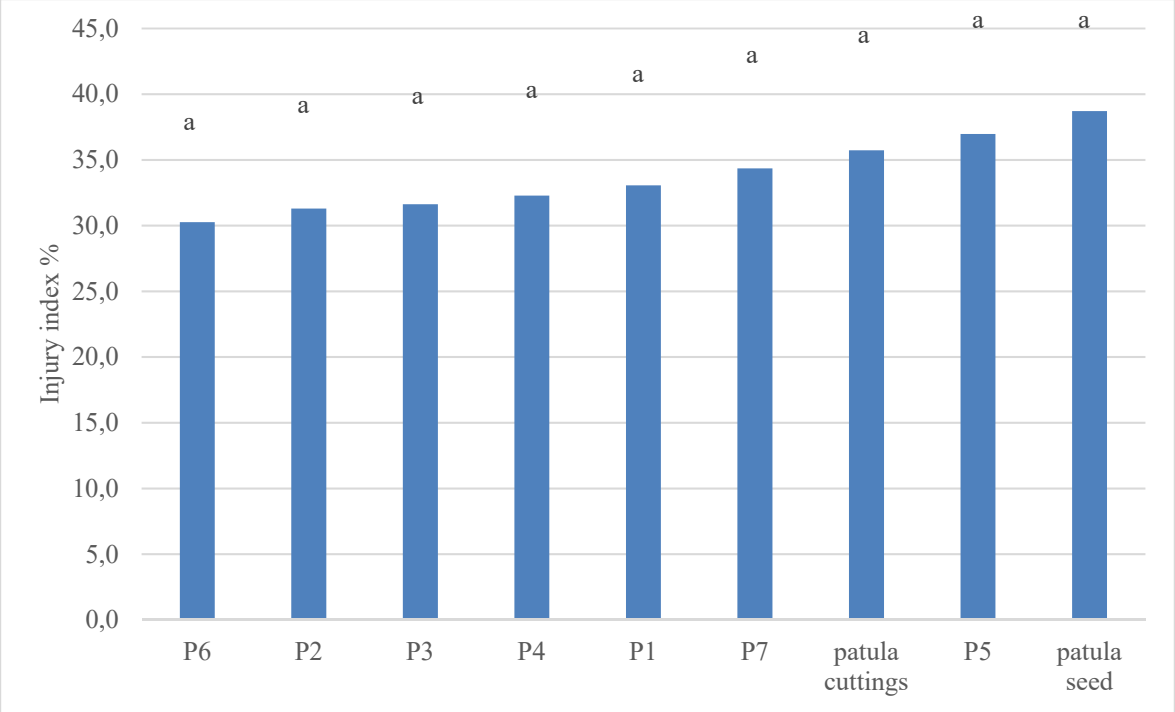

Figure 4.7: Comparison of the mean I_t of the selections of patula seed versus cuttings screened during the EL experiment across the two target temperatures of -3 and -6°C (standard deviation bars with the same letter does not differ significantly, p = 018, r^2 = 0.059, n = 6)

4.3.3. Interspecific hybrids

4.3.3.1. Constant temperatures

The I_t at -9°C and -12°C were in general more than 50% and only significant differences at -3 and -6°C will be reported (Figure 4.8). At -3°C only hybrids of PECH differed significantly (p <0.0001and r^2 =0.94) from the PPTH, PPTL and PPPOH hybrids. However, at -6°C, all the interspecific hybrids did not differ significantly (p < 0.0001 and r^2 = 0.91) from each other (Table 4.7).

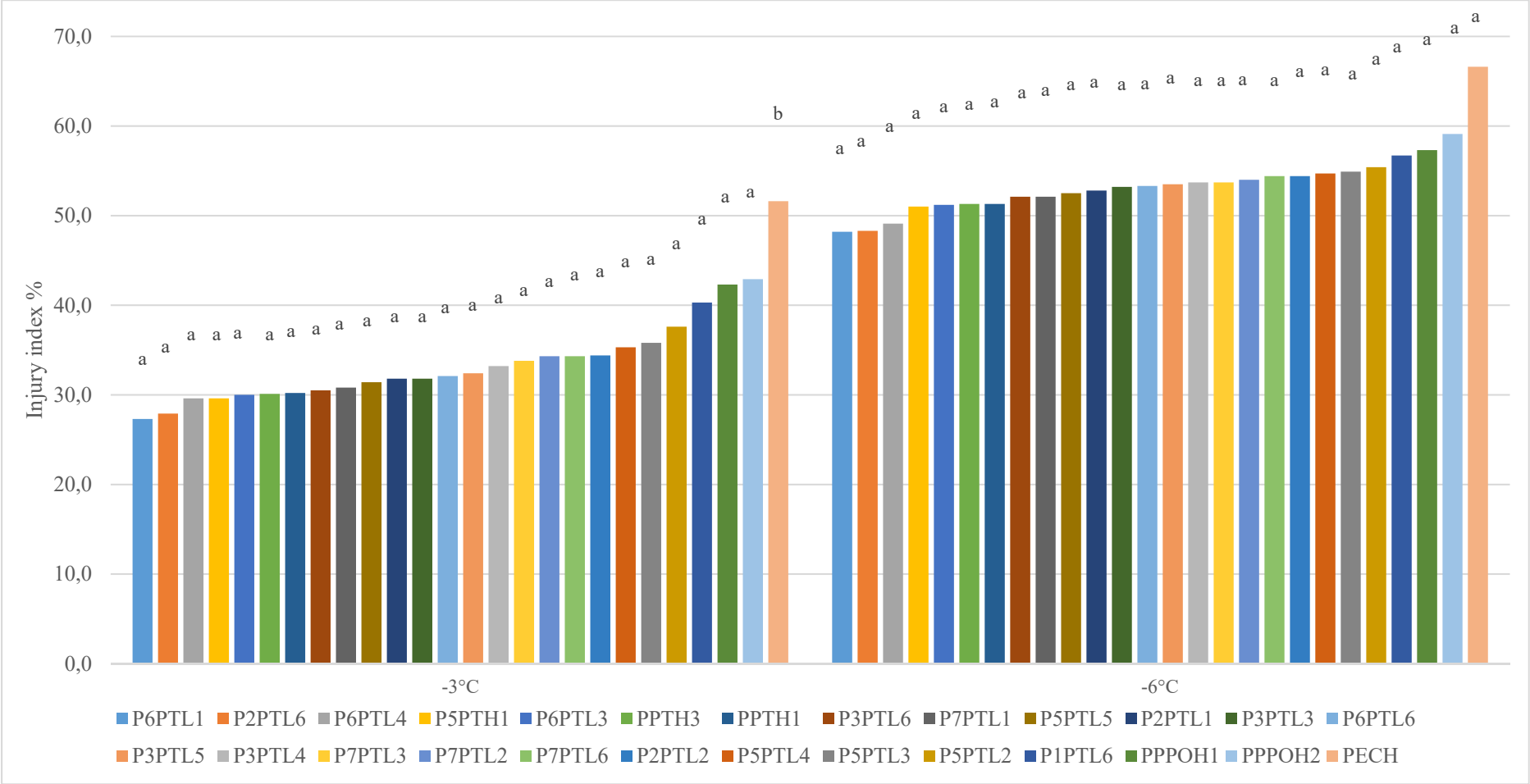

Figure 4.8: Mean I_t for interspecific hybrids per family screened during the EL experiment per target temperatures of -3 and -6°C (standard deviation bars with the same letters does not differ significantly, $p = 0.15$, $r^2 = 0.059$, $n = 6$)

Table 4.7: Mean I_t for interspecific hybrids per family screened during the EL experiment per target temperatures of -3 and -6˚C (standard deviation bars with the same letters does not differ significantly)

Seed or cuttings	**-3 (23.3 ± 2.8) [a]**	**-6 (44.7 ± 3.1) [b]**
PECH	[2] 51.6 ± 0.8 [b]	[3] 66.6 ± 0.76 [a]
$PPPOH_1$	[2] 42.3 ± 3.6 [a]	[2] 55.2 ± 0.7 [a]
$PPPOH_2$	[2] 42.9 ± 4.7 [a]	[2] 57.3 ± 3.5 [a]
$PPTH_3$	[1] 30.2 ± 4.6 [a]	[2] 53.2 ± 3.1 [a]
$PPTH_1$	[1] 30.1 ± 4.3 [a]	[2] 51.3 ± 3.6 [a]
$P1PTL_6$	[2] 40.3 ± 2.2 [a]	[2] 54.4 ± 1.6 [a]
$P2PTL_1$	[1] 31.8 ± 5.0 [a]	[2] 48.3 ± 1.9 [a]
$P2PTL_2$	[1] 34.4 ± 4.9 [a]	[2] 53.5 ± 1.9 [a]
$P2PTL_6$	[1] 27.9 ± 6.3 [a]	[2] 51.3 ± 3.5 [a]
$P3PTL_3$	[1] 31.8 ± 3.5 [a]	[2] 53.7 ± 1.9 [a]
$P3PTL_4$	[1] 33.2 ± 3.7 [a]	[2] 51 ± 3.1 [a]
$P3PTL_5$	[1] 32.4 ± 1.1 [a]	[2] 52.1 ± 1.8 [a]
$P3PTL_6$	[1] 30.5 ± 6.4 [a]	[2] 55.4 ± 8.4 [a]
$P5PTH_1$	[1] 29.6 ± 6.3 [a]	[2] 54.9 ± 3.2 [a]
$P5PTL_2$	[1] 37.6 ± 6.6 [a]	[2] 54.7 ± 2.2 [a]
$P5PTL_3$	[1] 35.8 ± 7.1 [a]	[2] 48.2 ± 7.2 [a]
$P5PTL_4$	[1] 35.3 ± 6.5 [a]	[2] 52.8 ± 1.2 [a]
$P5PTL_5$	[1] 31.4 ± 1.4 [a]	[2] 51.2 ± 5.1 [a]
$P6PTL_1$	[1] 27.3 ± 0.2 [a]	[2] 49.1 ± 2.4 [a]
$P6PTL_3$	[1] 30.0 ± 0.9 [a]	[2] 53.3 ± 2.6 [a]
$P6PTL_4$	[1] 29.6 ± 3.7 [a]	[2] 52.5 ± 1.8 [a]
$P6PTL_6$	[1] 32.1 ± 4.3 [a]	[2] 54.0 ± 2.8 [a]
$P7PTL_1$	[1] 30.8 ± 2.6 [a]	[2] 54.4 ± 3.1 [a]
$P7PTL_2$	[1] 34.3 ± 4.6 [a]	[2] 53.7 ± 1.9 [a]
$P7PTL_3$	[1] 33.8 ± 5.5 [a]	[2] 56.7 ± 2.1 [a]
$P7PTL_6$	[1] 34.3 ± 4.6 [a]	[2] 59.1 ± 3.2 [a]

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

Means in rows with the same letter does not differ significantly

4.3.3.2. Fluctuations in temperatures

PECH had a higher I_t value than the other hybrids at -3 and -6°C (Table 4.7, 4.8). $PPPOH_1$ and $PPPOH_2$ had a moderate susceptibility to frost at -3 and -6°C, while PPTH hybrids had a better survival rate at -3 and -6°C. PPTL hybrids had I_t value less than 40% at -3°C (tolerant to frost), while moderate tolerant at -6°C. All the PPTL and PPTH hybrids were more tolerant than PECH. P_6PTL_4 differed significantly (p =0.15, r^2=0.64) from the other hybrids as it had an I_t of less than 40% (indicating tolerance to frost). PECH had I_t values more than 60% (susceptible to frost), while PPTH, PPTL, $PPPOH_1$, $PPPOH_2$ had I_t values between 40 and 60% (moderate tolerance to frost).

Table 4.8: Comparison of the mean I_t of the interspecific hybrids per family (left to right in rows) screened across target temperatures during the EL experiment at target temperatures of -3, -6, -9 and -12°C (n = 6)

Species name	Target temperature (°C)			
	-3 (23.3 ± 2.8) [A]	**-6 (44.7 ± 3.1) [B]**	**-9(65.1±3.5 [c])**	**-12 (69.7±3.3 [c])**
PECH	[2]51.6 ± 0.8	[3]66.6 ± 0.76	[3] 72.6 ± 3.3	[3] 73.9 ± 1.5
PPPOH1	[2]42.3 ± 3.6	[2]55.2 ± 0.7	[3] 69.2 ± 2.2	[3] 72.1 ± 0.7
PPPOH2	[2]42.9 ± 4.7	[2]57.3 ± 3.5	[3] 71.1 ± 2.8	[3] 71.6 ± 1.3
PPTH3	[1]30.2 ± 4.6	[2]532. ± 3.1	[3] 60.1 ± 4.7	[3] 63.3 ± 0.3
PPTH1	[1]30.1 ± 4.3	[2]51.3 ± 3.6	[3] 60.4 ± 0.6	[3] 60.8 ± 0.6
P1PTL6	[2]40.3 ± 2.2	[2]54.4 ± 1.6	[3] 62.3 ± 1.4	[3] 69.9 ± 0.4
P2PTL1	[1]31.8 ± 5.0	[2]48.3 ± 1.9	[3] 65.5 ± 3.9	[3] 69.5 ± 1.0
P2PTL2	[1]34.4 ± 4.9	[2]53.5 ± 1.9	[3] 65.3 ± 3.6	[3] 70.5 ± 1.1
P2PTL6	[1]27.9 ± 6.3	[2]51.3 ± 3.5	[3] 65.2 ± 2.6	[3] 71.9 ± 1.7
P3PTL3	[1]31.8 ± 3.5	[2]53.7 ± 1.9	[3] 65.2 ± 4.3	[3] 71.5 ± 1.3
P3PTL4	[1]33.2 ± 3.7	[2]51 ± 3.1	[3] 63.8 ± 3.3	[3] 72.1 ± 3.0
P3PTL5	[1]32.4 ± 1.1	[2]52.1 ± 1.8	[3] 64.2 ± 6.2	[3] 70.3 ± 1.0
P3PTL6	[1]30.5 ± 6.4	[2]55.4 ± 8.4	[3] 61.1 ± 1.3	[3] 69.7 ± 0.6
P5PTH1	[1]29.6 ± 6.3	[2]54.9 ± 3.2	[2] 59.5 ± 6.1	[3] 63.9 ± 1.0
P5PTL2	[1]37.6 ± 6.6	[2]54.7 ± 2.2	[3] 63.4 ± 4.5	[3] 71.4 ± 1.2
P5PTL3	[1]35.8 ± 7.	[2]49.1 ± 2.4	[3] 65.3 ± 24.5	[3] 72.1 ± 1.1
P5PTL4	[1]35.3 ± 6.5	[2]52.8 ± 1.2	[3] 64.8 ± 5.5	[3] 71.6 ± 1.9
P5PTL5	[1]31.4 ± 1.4	[2]51.2 ± 5.1	[3] 64.8± 5.7	[3] 71.0 ± 1.1
P6PTL1	[1]27.3 ± 0.2	[2]48.2 ± 7.2	[3] 64.4 ± 7.1	[3] 66.2 ± 3.3
P6PTL3	[1]30.0 ± 0.9	[2]53.3 ± 2.6	[3] 62.4 ± 2.9	[3] 70.3 ± 0.4
P6PTL4	[1]29.6 ± 3.7	[2]52.5 ± 1.8	[3] 63.7 ± 3.6	[3] 70.7 ± 1.6
P6PTL6	[1]32.1 ± 4.3	[2]54.0 ± 2.8	[3] 61.5 ± 3.7	[3] 71.6 ± 2.1
P7PTL1	[1]30.8 ± 2.6	[2]54.4 ± 3.1	[3] 64.8 ± 4.7	[3] 69.9 ± 1.6
P7PTL2	[1]34.3 ± 4.6	[2]53.7 ± 1.9	[3] 65.3 ± 3.6	[3] 70.5 ± 1.1
P7PTL3	[1]33.8 ± 5.5	[2]56.7 ± 2.1	[3] 65.2 ± 5.1	[3]70.7 ± 1.1
P7PTL6	[1]34.3 ± 4.6	[2]59.1 ± 3.2	[3] 60.9 ± 1.7	[3] 68.9 ± 1.3

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

Means in rows with the same letter does not differ significantly

4.3.3.3. Species versus fluctuations in temperatures

Comparison of the I_t of selections screened during the EL experiment for both -3 and -6°C indicated that PECH differed significantly ($p = 1.0$, $r^2 = 0.05$) from the other hybrids between interspecific hybrids (Figure 4.9). Frost tolerance was ranked from high (P_1PTH_3), moderate (other PPTL and PPTH hybrids) to low (PECH, $PPPOH_1$ and $PPPOH_2$).

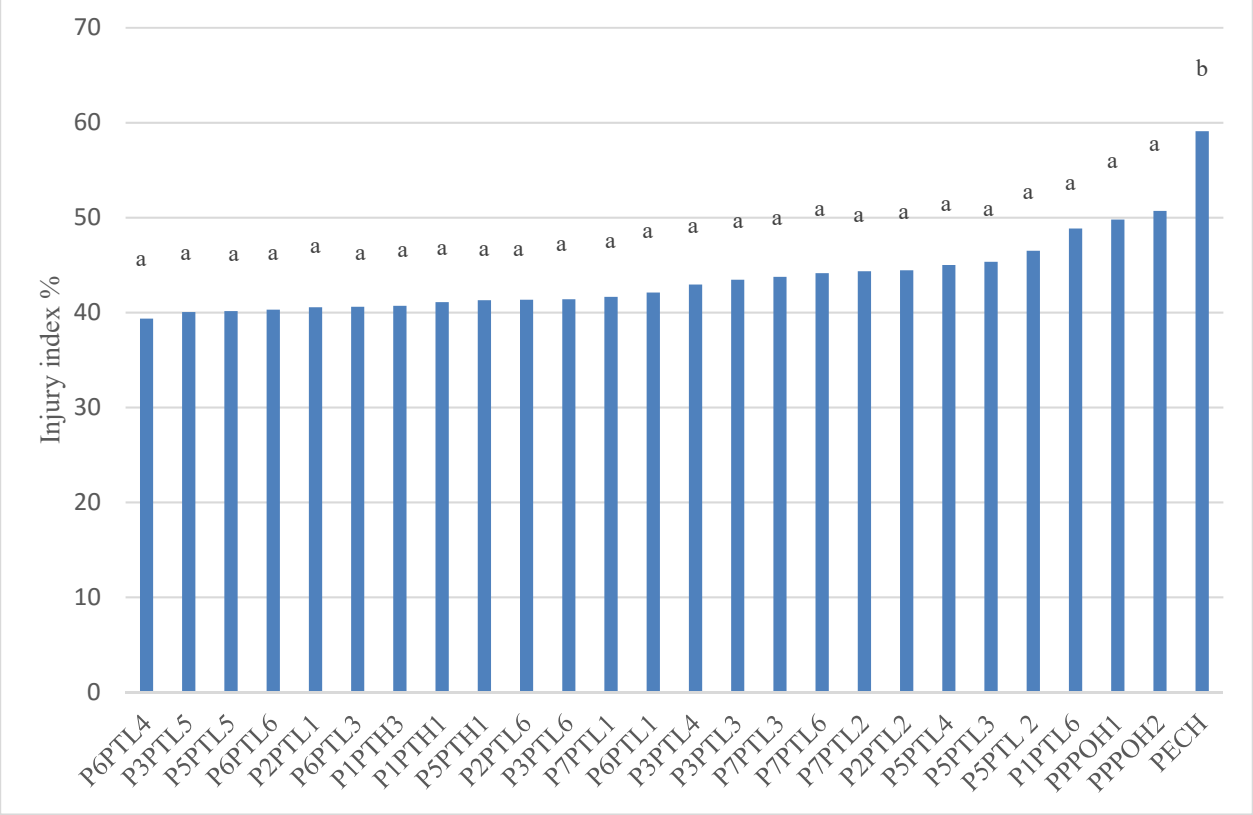

Figure 4.9: Comparison of the mean I_t of hybrids screened during the EL experiment across the target temperatures -3 and -6°C (standard deviation bars with the same letter does not differ significantly, $p = 0.06$, $r^2 = 0.59$, $n = 6$)

All the PTH hybrid families of interspecific hybrid were grouped together according to the female parent (Figure 4.10) to determine significant differences between groups. Hybrid groups were ranked as follows according to significant differences ($p = 0.22$, $r^2 = 0.47$). P_1PTH and P_6PTL were tolerant to frost, P_5PTL, P_2PTL, P_3PTL, P_7PTL, P_5PTH and P_1PTL had a moderate tolerance to frost while PPPOH and PECH were susceptible to frost at both -3 and -6°C.

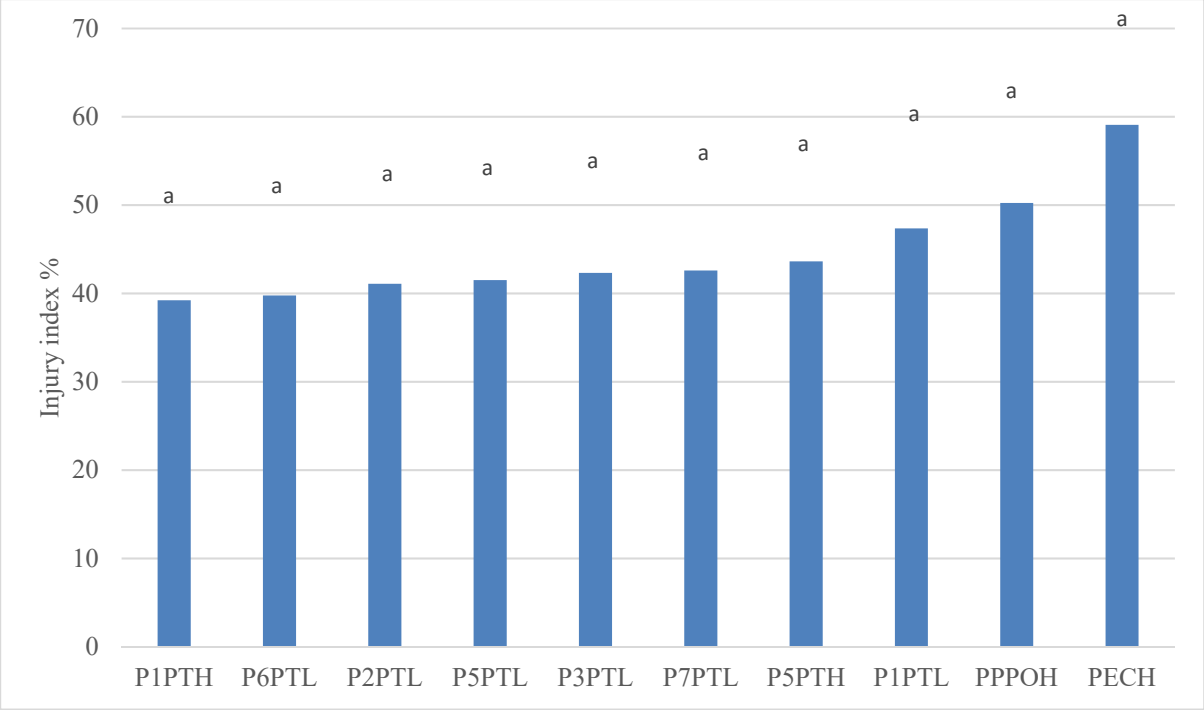

Figure 4.10: Comparison of the mean I_t of the hybrids screened during the EL experiment across the target temperatures of -3 and -6°C (standard deviation bars with the same letter does not differ significantly, $p = 0.22$, $r^2 = 0.47$, $n = 6$)

4.3.3.4. Summary of hybrid results

All the families of an interspecific hybrid were grouped together to determine significant differences between groups at -3 and -6°C (Figure 4.11) and combined target temperatures (Figure 4.12). There were significant differences between the four interspecific hybrids tested in the study in terms of frost tolerance at -3 and -6°C separately ($p < 0.0001$and $r^2 = 0.71$) and combined ($p = 090$ and $r^2 = 0.27$) PPTH, PPTL and PPPOH did not differ significantly from each other but differed significantly ($p = 0.47$, $r^2 = 0.43$) PECH. In addition, PPTH and PPTL had I_t values of less than 40% (indicating tolerance to frost) at -3°C and moderate tolerant to frost at -6°C (Table 4.9). However, at the combined temperatures (-3 and -6°C), only PPTH had an I_t of less than 40%.

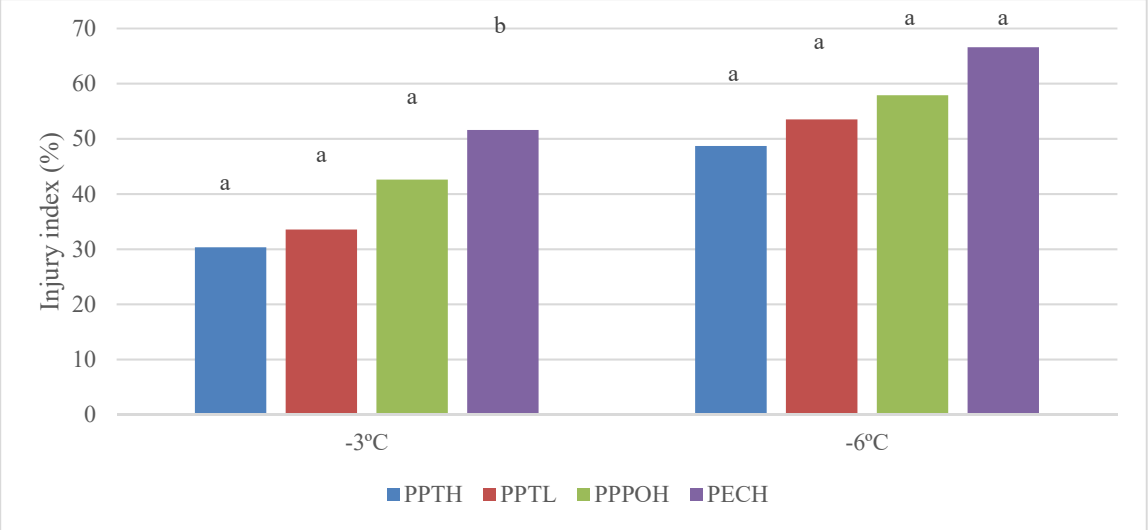

Figure 4.11: Comparison of the mean I_t for all the selections screened during the needle experiment per target temperatures of -3 and -6°C (standard deviation bars with the same letter does not differ significantly, $p = 0.17$, $r^2 = 0.48$ $n = 6$)

Table 4.9: Comparison of the mean I_t for all the selections screened during the needle experiment per target temperatures of -3 and -6°C (standard deviation bars with the same letter does not differ significantly, $n = 6$)

Hybrids	**Target temperature (ºC)**	
	-3 (23.3 ± 2.8 [a])	**-6 (44.7 ± 3.1 [b])**
PPTH	[1] 30.3 ± 3.1 [a]	[2] 48.7 ± 7.9 [a]
PPTL	[1] 33.8 ± 4.9 [a]	[2] 53.5 ± 16.7 [a]
PPPOH	[1] 42.6 ± 4.7 [a]	[2] 57.9 ± 14.2 [a]
PECH	[1] 51.6 ± 3.6 [b]	[2] 66.6 ± 5.6 [a]

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

Means in rows with the same letter does not differ significantly

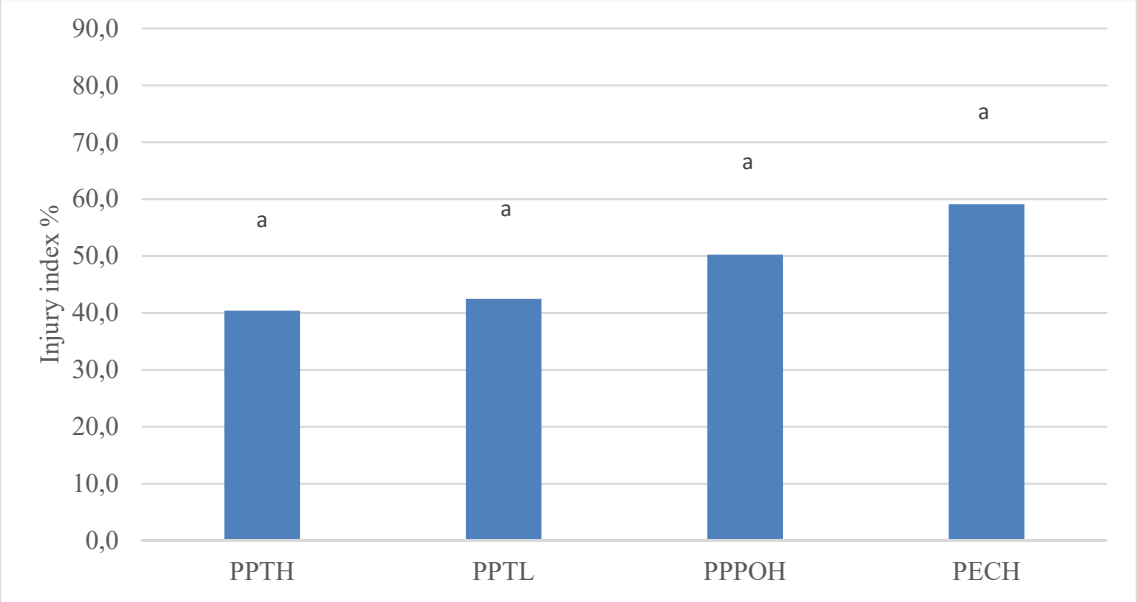

Figure 4.12: Comparison of the mean I_t for all the hybrids screened during the EL experiment per target temperatures of -3 and -6°C (standard deviation bars with the same letter does not differ significantly, $p = 0.17$, $r^2 = 0.48$, $n = 6$)

There were significant difference ($p < 0.0001$, $r^2 = 0.48$) in frost tolerance between patula and the interspecific hybrids screened (combined families) at -3 and -6°C separately (Figure 4.13) and combined (Figure 4.14). Patula was more tolerant than the interspecific hybrids with an I_t of less than 40% (tolerant to frost). PPTH and PPTL only differed significantly ($p = 0.0009$, $r^2 = 0.68$) at -6°C and the combined target temperatures -3 and -6°C ($p = 0.43$, $r^2 = 0.47$). PPPOH and PECH differed significantly from each other with an I_t between 40 and 60% (moderate tolerant to frost) at -3°C but susceptible to frost at -6°C.

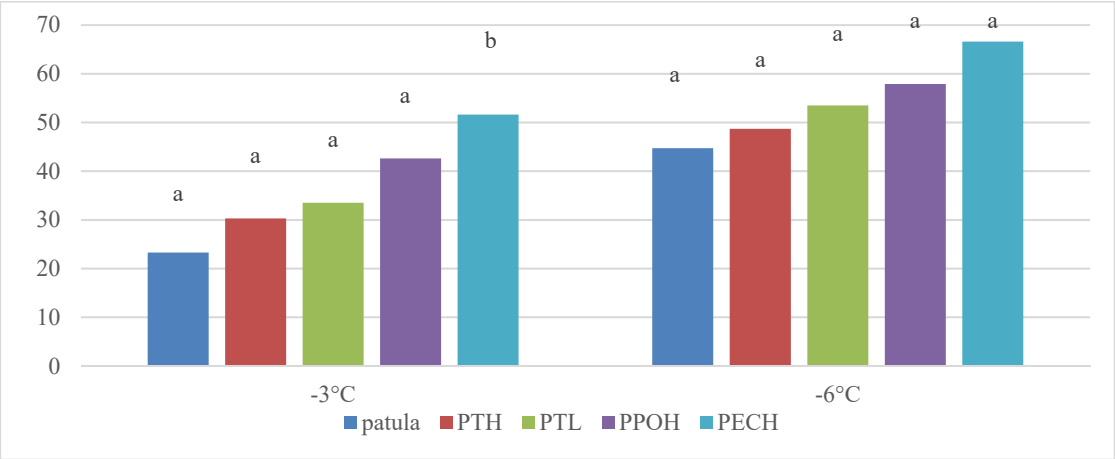

Figure 4.13: Comparison of the mean I_t for all the hybrids and patula screened during the EL experiment per target temperatures of -3 and -6°C (standard deviation bars with the same letter does not differ significantly, $p = 0.30$, $r^2 = 0.48$ $n = 6$)

Table 4.10: Comparison of the mean I_t for all the selections screened during the needle experiment per target temperatures of -3 and -6°C (standard deviation bars with the same letter does not differ significantly, $n = 6$)

Hybrids	**Target temperature (ºC)**	
	-3 (23.3 ± 2.8 [a])	**-6 (44.7 ± 3.1 [b])**
Patula	[1] 23.1 ± 0.9 [a]	[2] 44.7± 16.7 [a]
PPTH	[1] 30.3 ± 3.1 [a]	[2] 48.7 ± 7.9 [a]
PPTL	[1] 33.5 ± 4.9 [a]	[2] 53.5 ± 16.7 [a]
PPPOH	[1] 42.6 ± 4.7 [a]	[2] 57.9 ± 14.2 [a]
PECH	[1] 51.6 ± 3.6 [b]	[2] 66.6 ± 5.6 [a]

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

Means in rows with the same letter does not differ significantly

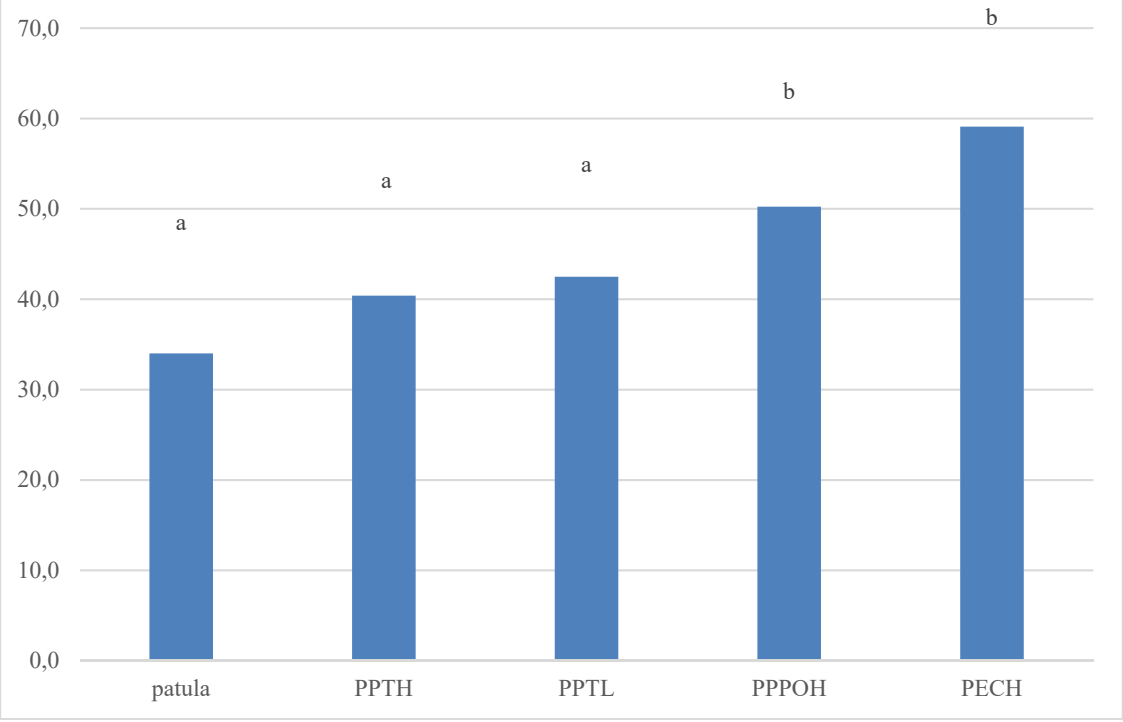

Figure 4.14: Comparison of the mean I_t for hybrids and patula screened during the EL experiment at target temperatures of -3 and -6°C (standard deviation bars with the same letter does not differ significantly, $p = 0.12$, $r^2 = 0.47$, $n = 6$)

4.4. Whole plant experiment

4.4.1. Pure species

At the target temperatures -9 and -12°C (Table 4.11) all the species scored a 3 (susceptible to frost). However, at -6°C only greggii was tolerant to frost (score of 1), while patula (seed and cuttings), elliottii, maximinoi and PTH scored 2 (moderate tolerant to frost). The remaining species had scores of 3, indicating susceptible to frost. Only caribaea had a score of 3 (susceptible to frost) at -3°C, while maximinoi, oocarpa, taeda and PTL had a score of 2 (moderate tolerant to frost). Patula, elliottii, greggii and PTH scored 1 (tolerant to frost) at -3°C.

Table 4.11: Comparison of the mean I_t for pure species screened (left to right in rows) at target temperatures of 3, -6, -9 and -12°C (n = 3)

Species name	**Target temperature (°C)**				**Average**
	-3	**-6**	**-9**	**-12**	
caribaea	3	3	3	3	3
patula seed	1	2	3	3	2.3
patula cuttings	1	2	3	3	2.3
elliottii	1	2	2	3	2.3
greggii	1	1	3	3	2.7
maximinoi	2	2	3	3	2.5
oocarpa	2	3	3	3	2.8
taeda	2	3	3	3	2.8
PTH	1	2	3	3	2.3
PTL	2	2	3	3	2.5
Average	1.5	2.1	3	3	

[1]= tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

4.4.2. Patula seed versus patula cuttings

As with the pure species, at target temperatures -9°C, P_1, P_2 and P_6 scored 2, while at -12°C all the patula families scored 3 (susceptible to frost) (Table 4.12). However, at -3°C all the patula families scored 1 (tolerant to frost), while at -6°C patula (seed and cuttings), P_4, P_5 and P_7 scored 2 (moderate tolerant to frost).

Table 4.12: Comparison of the mean I_t for selections of patula seed and cuttings screened (from left to right in rows) during the WPFT at target temperatures of -3, -6, -9 and -12°C

Patula family	**Target temperature (ºC)**				**Average**
	-3	**-6**	**-9**	**-12**	
patula cuttings	1	2	3	3	2.3
patula seed	1	2	3	3	2.3
P1	1	1	2	3	2.3
P2	1	1	2	3	2.3
P3	1	2	3	3	2.3
P4	1	2	3	3	2.3
P5	1	2	3	3	2.3
P6	1	1	2	2	2.3
P7	1	2	3	3	2.3
Average	1	1.7	2.7	2.9	

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

4.4.3. Interspecific hybrids

The same trends were evident as with the patula cuttings versus patula seedlings. All the patula hybrids scored 3 (susceptible to frost) at -9 and -12°C (Table 4.13). At -6°C all the patula hybrids scored 2 (moderate tolerant to frost), while at -3°C all the patula hybrids scored 1 (tolerant to frost). PECH scored 3 at all four target temperatures, while PPPOH scored 2 (moderate tolerant to frost) at -3 and -6°C. At -9 and -12°C, PPPOH hybrids scored 3 (susceptible to frost).

Table 4.13: Comparison of survival score for interspecific hybrids per family screened (left to right in rows) during the WPFT experiment at target temperatures of -3, -6, -9 and -12°C (*n* =3)

Hybrid	**Target temperature (°C)**				**Average**
	-3°C	**-6°C**	**-9°C**	**-12°C**	
PECH	3	3	3	3	3
$PPPOH_1$	2	2	3	3	2.5
$PPPOH_2$	2	2	3	3	2.5
P_1PTH_1	1	2	3	3	2.3
P_1PTH_3	1	2	3	3	2.3
P_1PTL_6	2	2	3	3	2.5
P_2PTL_1	1	2	3	3	2.3
P_2PTL_2	1	2	3	3	2.3
P_2PTL_6	1	2	3	3	2.3
P_3PTL_3	1	2	3	3	2.3
P_3PTL_4	1	2	3	3	2.3
P_3PTL_5	1	2	3	3	2.3
P_3PTL_6	1	2	3	3	2.3
P_5PTH_1	1	2	3	3	2.3
P_5PTL_2	1	2	3	3	2.3
P_5PTL_3	1	2	3	3	2.3
P_5PTL_4	1	2	3	3	2.3
P_5PTL_5	1	2	3	3	2.3
P_6PTL_1	1	2	3	3	2.3
P_6PTL_3	1	2	3	3	2.3
P_6PTL_4	1	2	3	3	2.3
P_6PTL_6	1	2	3	3	2.3
P_7PTL_1	1	2	3	3	2.3
P_7PTL_2	1	2	3	3	2.3
P_7PTL_3	1	2	3	3	2.3
P_7PTL_6	1	2	3	3	2.3
Average	1.2	2	3	3	

[1] = tolerant, [2] = moderate tolerance to frost and [3] = susceptible to frost

4.5. Correlations

There was a significant correlation ($p < 0.0001$) between the EL and WPFT techniques at all four target temperatures -3, -6, -9 and -12°C (Table 4.9). A strong positive correlation was evident between the pure species and interspecific hybrids at all 4 target temperatures. At -3°C both pure species and hybrids families had a medium positive correlation, at -6°C pure species had a medium positive correlation while the hybrid families had a weak positive correlation. At -9°C, pure species had a medium positive correlation while the hybrid families had a weak positive correlation. At -12°C pure species had a weak positive correlation while the correlation for hybrid families was not estimated.

Table 4.14: Correlation (Pearson correlation coefficient) between the I_t of the EL and WPFT experiments for pure species and interspecific hybrids at all four target temperatures ($p<.0001$, $r^2= 0.64$, $n = 3$)

Target temperature	Species/hybrid	Correlation coefficient	Comment
-3, -6, -9 and -12°C	All species and hybrid families	0.81	strong positive
	Pure species	0.79	strong positive
	Hybrid families	0.81	strong positive
-3°C	Pure species	0.69	medium positive
	Hybrid families	0.31	medium positive
-6°C	Pure species	0.64	medium positive
	Hybrid families	0.26	weak positive
-9°C	Pure species	0.38	medium positive
	Hybrid families	0.25	weak positive
-12°C	Pure species	0.21	weak positive
	Hybrid families	0.21	Weak positive

CHAPTER FIVE

DISCUSSION

5.1. Introduction

Patula is one of the dominant softwood species planted in South Africa because of its good growth, wood and pulp properties as well as good frost tolerance. However, this species is highly susceptible to *F. circinatum.* Patula has been hybridized with species like PTH, PTL and oocarpa to improve *F. circinatum* tolerance. Field results, however, have indicated that these hybrids are more susceptible to frost causing damage to trees in the field (Mitchell *et al*., 2012). Previous studies also indicated that PTH and PTL hybridises easily with patula for improved tolerance to *F. circinatum* (Roux *et al*., 2007) but with a lower frost tolerance than patula.

5.2. Patula seed vs cuttings

In general, there was no significant difference in frost tolerance between patula seedlings and patula cuttings for both the EL and WPFT experiments in this study. Patula cuttings are more woody and have more secondary needles compared to seedlings, and are also older plants as they take 50% longer to produce in the nursery. Patula cuttings (mixed families) were therefore also older with bigger needles size and twice the length than the patula seedlings, and was expected to have increased frost tolerance compared to seedlings. Previous studies on other traits such as *F. circinatum* indicated no significant differences between seedlings and cuttings of various species and hybrids tested (Mitchell *et al*., 2012) and this study seem to indicate that it also applies to frost tolerance screening. Studies on the tolerance of PTL and other pine hybrids to *F. circinatum* in greenhouse trials indicated that cuttings were more tolerant to *F. circinatum* than seedlings (Zagory & Libby, 1985 & Mitchell *et al*., 2004). Furthermore, *P. radiata* cuttings were more tolerant to *Endocronartium harknessii* (Power *et al*., 1994) and taeda cuttings showed more tolerance to *Cronartium quercuum* than seedlings (Frampton & Goldfarb, 2000). This might be due to the increased maturity of the cuttings opposed to the seedlings at the same age (Zagory & Libby, 1985).

During this study, the seven patula families (all seedlings) were compared and there were no significant differences between the families at -3 and-6°C. Therefore, a more comprehensive experimental design needs to be tested with equal number and similar patula families as cuttings and seedlings. The number of repetitions can also be increased from 6 to 10 to eliminate possible experimental error between patula families.

5.3. Pure species

All the pure species experienced severe frost damage at -12°C, while greggii had a moderate tolerance to frost at -9°C for both EL and WPFT. At -6°C for EL, greggii and elliottii were tolerant to frost, while taeda, PTH and maximinoi had a moderate tolerance to frost. PTL, oocarpa and caribaea (EL) were the only species to be susceptible to frost at -6°C. At -3°C, PTH, maximinoi, PTL, oocarpa and caribaea (EL) had a moderate tolerance to frost, while the other species were tolerant to frost. Previous studies also indicated that caribaea is more susceptible to frost than other tropical species like elliottii (Hodge *et al*., 2012). The reason for high damage in caribaea was because this species cannot tolerate temperatures below 0°C as indicated in Table 2.1. Therefore, this species should not be planted in frost prone areas unless it is hybridized with a frost tolerant species like elliottii.

In vivo studies in South Africa were conducted on PTH and PTL families (mature trees) subjected to several hours at temperatures between -2 and -3°C. PTH families remained alive and green, while the PTL families turned brown and died (Donahue, 1993). Other studies (EL) indicated that PTL cannot tolerate temperatures lower than 0°C (Dvorak *et al*., 2000a). *In vitro* (EL) frost tolerant studies on PTH or PTL indicated that PTH was more tolerant to frost at temperatures -7, -14 and -21°C (Hodge & Dvorak, 2012). PTH can tolerate a minimum temperature of -3°C in its native stage, while oocarpa (seedlings) can tolerate up to -5°C (Dvorak, 1985). Therefore, the minimum temperatures at which a species can tolerate should be considered before planting in frost prone areas (Picchi & Barrett, 1967). Previous studies where oocarpa trees were planted in frost prone areas indicated that one year-old oocarpa seedlings were killed by heavy frost (-8°C) in Brazil (Picchi & Barrett, 1967).

Maximinoi (seedlings) can tolerate minimum temperatures between -2 and -3°C (Dvorak, 1985). Previous studies on susceptibility and of various provenances of PTL and maximinoi indicated that field survival of maximinoi seedlings were lower than that of PTL at -3°C. Therefore, this species should also not be planted in frost prone areas (Mitchell *et al*., 2012). Taeda, however, can tolerate minimum temperatures of -18°C (Dvorak, 1985). Hodge *et al.* (2012) did a study on artificial freezing of tropical and temperate species. Results indicated that greggii, elliottii, taeda and PTH had a better frost tolerance than other species. Previous studies indicated that when hybridizing patula, the better species choice could be PTH, PTL and oocarpa due to the *F. circinatum* tolerance of these species (du, Toit, 2012).

5.3.1. Tolerance of greggii to frost

Studies on mature trees of patula in South Africa, indicated that patula had better frost tolerance than greggii, although these two species sometimes perform equally *in vivo* (Mitchell *et al*., 2004). Hodge *et al*. (2012) also indicated that greggii north and patula appear to have equal frost tolerance, but it can vary depending on the planting site and temperature. Previous studies (EL with needles) on greggii provenances indicated that greggii north can tolerate up to -18°C and greggii south up to -12°C (Aldrete *et al*., 2008). Previous studies on seedlings (WPFT) indicated that greggii north had better frost tolerance, however, bud set and initiated bud break were earlier than greggii south (Kuser & Ching, 1980). Patula (seedlings) can tolerate minimum temperatures between -4 and -12°C (Dvorak, 1985), while Hodge *et al*. (2012) indicated that patula, greggii and elliottii were more frost tolerant at -7, -14 and -21°C. Although previous studies indicated that greggii might be a better plantation species than patula and elliottii due to the species tolerance to frost (Mitchell *et al*., 2012), greggii cannot be hybridised with patula as it is susceptible to *F. circinatum* (Dvorak *et al*., 2000a, Roux *et al*., 2007). Therefore, pure greggii north can be planted in frost prone areas in South Africa in some areas previously planted with patula since the hybrid between greggii and patula cannot work as both species are susceptible to *F. circinatum*.

Hodge *et al.* (2012) ranked *Pinus* species according to their tolerance to frost as follows: susceptible to frost were caribaea, oocarpa and PTL, moderate tolerance to frost were *P. patula* var. *patula* and greggii south and frost tolerance were greggii north, elliottii and taeda. These results were in line with EL and WPFT experiments performed during this study. However, some of the results from Hodge *et al.* (2012) contradict the results obtained in this study for example, the order of frost tolerance of the species in this study was greggii, patula, elliottii and taeda for both EL and WPFT. Patula, PTH and PTL could be the most promising species to hybridise. This is due to tolerance of all PTL families to *F. circinatum,* while PTH families vary in their tolerance to *F. circinatum* but are more tolerant to *F. circinatum* than patula (Mitchell *et al*., 2011). PTH can be planted in cool temperate areas since it has frost tolerance while PTL can be planted on warm temperate areas due to its low frost tolerance, however, PTL has good *F. circinatum* tolerance of this species could be hybridized with patula and the hybrid could be planted in both cool and warm areas.

5.4. Interspecific hybrids

All the interspecific hybrids, except PECH and PPPOH, had a low I_t at -3°C during the EL experiments, indicating tolerance to frost. PECH and PPPOH were moderate tolerant to frost at -3°C, at -6°C PECH and PPPOH were susceptible to frost for the EL experiment. However, at -6°C, the I_t of all the interspecific hybrids (except PECH) were less than 55% indicating moderate tolerance to frost (EL). At

-9 and -12°C (EL) all the interspecific hybrids were susceptible to frost and had an I_t of more than 60%. Although the patula families performed the best at -3 and -6°C, interspecific hybrids with patula family P_6 performed the best at both -3 and -6°C (EL experiments). Previous studies used 50% as a cut-off indicating the highest I_t percent. In this study in order to compare *in vivo* and *in vitro* conditions three categories were used: first I_t values between 1-40% indicated frost tolerance, second, I_t values between 40 and 60% indicated moderate tolerance to frost and lastly, I_t values between 60 to 100% indicated susceptibility to frost.

The I_t for P_1PTH_1, P_1PTH_3 and P_5PTH_1 was less than 30% and approximately 49% at -3°C. At -6°C the I_t values for these hybrid families were below 50%. Interspecific hybrids P_1PTL_6, P_5PTL_2, P_5PTL_3 and P_5PTL_4 had an I_t of 35 to 40% and 49 to 54% at -3 and -6°C respectively. However, to study the I_t of interspecific hybrids between patula, PTL and PTH families better, a more complete factorial mating design will be needed. In general, PTL and PTH families did not differ significantly at -3 and -6°C although P_1PTH_1 had the lowest I_t at -3°C of 27%. PPPOH and PECH families had a higher I_t at both -3 and -6°C, although PPPOH had a better tolerance than PECH it was less tolerant compared to PPTH and PPTL for the EL experiments.

5.5. Frost tolerance of pure species vs interspecific hybrids

When all the hybrids are compared with the respective pure species (EL), the following ranking (high to low I_t) applies at both -3 and -6°C: greggii, elliottii, patula, taeda, PTH, PTL, PPTH, PPTL, PPPOH, PECH, oocarpa and caribaea. Interspecific hybrids (PPTH, PPTL, PPPOH and PECH) in this study performed intermediate between the two parents as indicated in previous studies (Lopez-Upton & Donahue, 1995; Dvorak *et al*., 2000a, Hodge *et al*., 2012). Although PPTH performed the best of all the interspecific hybrids screened in this study, it had slightly lower tolerance to *F. circinatum* than PTL (Dvorak *et al*, 2000b), lower wood density than patula (Miller, 1993). The reason for better tolerance of PPTH is that both parents (patula and tecunumanii) have frost tolerance, therefore pure species and hybrid (PPTH) can be planted in frost prone areas, PPTL and PPPOH lacks frost tolerance, thus, it is not suitable to be planted in frost prone areas since one parent of these hybrids (patula) offer frost tolerance. PECH on the other hand was significantly different from the other hybrids and cannot be planted in frost prone areas.

Another example of an interspecific hybrid with intermediate frost tolerance between the parent species is *P. patula* x *P. jaliscana* (Dvorak *et al*., 2000a). The genetic control of frost tolerance with a more comprehensive factorial mating design need to be investigated in further studies to assist with breeding objectives (pulp quality, frost tolerance and *F. circinatum* tolerance) and site species matching. Furthermore, the same patterns were evident during the WPFT than EL experiments.

5.6. Correlation between the EL and WPFT

Results obtained in this study between the EL and WPFT experiments indicated a strong positive correlation between all the selections (pure species and interspecific hybrids) and target temperatures (-3, -6, -9 and -12°C). Previous studies not only confirmed that the best methods to test frost tolerance are the EL and WPFT methods, but also indicated a positive correlation between these two methods (Glerum, 1976, Levitt, 1980, Hodge *et al*., 2012). The results from this study are in line with the artificial screening results obtained in the study by Hodge & Dvorak (2012). These two methods are also paramount in pre-screening nursery stock to limit losses in genetic material and assist with site species matching by determining frost tolerance levels and limit blanking costs. The methods are also fast, reliable, reputable and inexpensive (Glerum, 1976; Levitt, 1980, Hodge *et al*., 2012).

As *in vivo* conditions are not always as predictable as *in vitro* conditions (Sakai & Larcher, 1987), the combination of the two methods can provide valuable information on site species matching in terms of frost tolerance. The selections tested (pure species and interspecific hybrids) were planted in an *in vivo* trial during 2015 and 2016. However, warm winters in both years with no frost spells did not yield any results to compare seedlings in the nursery and *in vivo* conditions. For easy comparisons in the data, the same age seedlings (WPFT) and needles were used in both the EL and WPFT experiments during this study. Therefore, a new *in vivo* trial will be planted in 2017 to determine the correlation between nursery and *in vivo* planted seedlings.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

The two techniques (EL and WPFT) used in this study to screen various *Pinus* species and interspecific hybrid families, provided a good ranking for frost tolerance. The forestry industry can use these techniques to predict frost tolerance of new genotypes and hybrid combinations. For example, the results in this study and other artificial freezing studies indicated that PPTH is more frost tolerant than PPTL. The EL method can be used to screen various *Pinus* hybrids to determine within a short time period whether a hybrid is more or less tolerant than the parental species and specific parental families.

The EL and WPFT methods have proven to be the most reliable techniques on an operational scale as it is less time consuming and these two techniques were strongly positively correlated. The deployment of PPTH and PPTL hybrids are good alternatives to commercial planted patula due to improved growth, good wood and pulp properties, better tolerance to *F. circinatum* and improved post-planting survival. The PPTL hybrid, for example, is a viable replacement to patula especially within the warm temperate climate zone of South Africa. However, PTL and PTH hybrids cannot replace patula at all the high frost-prone site in South Africa as the frost tolerance is not as high as with patula. Therefore, good site species matching is essential when establishing PPTH, PPTL so sites which experience high levels of frost can be avoided. The PPTH hybrid is more tolerant to frost than PPTL and can thus be planted on a wider variety of sites, but it is still not as frost tolerant as patula.

Oocarpa, caribaea, PPPOH and PECH have little to no frost tolerance, therefore, these species should be planted in warm temperate zones. PTH has limited tolerance to frost, therefore this species can be used to replace patula and can be hybridised with patula and planted in frost prone areas. Greggii and elliottii on the other hand, are by far the most frost tolerant species. Therefore, these species can be planted in frost prone areas. There is however a drawback with the deployment of greggii north in South Africa. This species cannot be planted in South Africa because of its susceptibility to *F. cicrcinatum*. The species also has poor wood quality and it performs poorly on wet sites compared to patula.

In this study the EL and WPFT techniques indicated that not only can the *Pinus* species be evaluated for frost tolerance *in vivo, Pinus* species can also be tested *in vitro* to get results quicker. The results obtained between the two techniques indicated that there was a good correlation between the techniques. Even though there was a positive correlation between the two techniques, it would be important to have future studies that would extend the scoring system of plants after freezing from 0 to 5. Testing of cambium damage could also be used when scoring plants for survival in order to verify visual scoring

of plants. Also the EL techniques could be used on a wider scale to test many related PPTL and PPTH hybrid families in order to establish genetic inheritance of frost tolerance between the different families, this could also be used to determine genetic control of frost tolerance in other *Pinus* hybrid families.

Even though the results of this study supported the results obtained in previous studies that the PPTH (cooler sites) and PPTL (warmer sites) hybrids could be a viable taxon for replacement on sites planted with patula in South Africa. One of the major limitations in this study was the fact that there was not enough PTH families to test. Therefore, a large factorial mating design in order to test a large number of additional hybrid families should be included in future studies. In addition, field trials could be planted on more than one frost prone site in order to verify the *in vitro* results. In this study field trials were only planted in KZN, Pinewoods plantation. Unfortunately no frost was experienced during this study, therefore, the result of this study can only be verified during the year in which frost will occur.

The following recommendations are proposed:

- There is variation in frost tolerance of PPTH families, therefore, future studies should include more PTH families in a factorial mating design in order to select the best PTH families to hybridise with patula.
- Advance generation crosses among the selected hybrids might improve frost tolerance.
- It is important that *in vitro* screening for frost tolerance be done before the establishment of field trials to determine the I_t and make informed decisions.
- As the circadian model (developed for Pinewoods plantation from June to August) indicate the lowest average temperature documented were -3°C, *in vitro* experiments conducted at -3°C can already be used to assist with breeding objectives and site species matching. However, -6°C is a more representable of *in vivo* conditions and future experiments.
- The strong positive correlation between the EL and WPFT experiments confirmed that the EL method is a reliable technique to test frost tolerance and should be applied.
- It is further recommended that climatic data from several seasons at specific frost prone sites be used to determine at what temperatures frost damage occurs. This information can be used to alter or confirm target temperatures used during the *in vitro* screening.
- Secondary (not primary) needles should be harvested and tested, as they are less sensitive to freezing resulting in more accurate results as it is more similar to recently established seedlings.

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